The Ecology of *Staphylococcus aureus*

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by Andrew Benjamin Libberton

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Abbreviations

ACC	*Corynebacterium accolens*
AUR	*S. aureus*
BAC	*Bacillus* sp.
BRE	*Brevidobacterium sp.*
BSI	*Bacillus simplex*
CAP	*S. capitis*
CFR	*Citrobacter freundii*
CKO	*Citrobacter koseri*
COY	*Corynebacterium coyleae*
EAE	*Enterobacter aerogenes*
ECO	*Escherichia coli*
EPI	*S. epidermidis*
EQU	*S. equorum*
GHA	*Gemella haemolysans*
HAE	*S. haemolyticus*
HOM	*S. hominis*
KKR	*Kocuria kristinae*
LEN	*S. lentus*
LUG	*S. lugdunensis*
LUT	*Micrococcus luteus*
MAC	*Corynebacterium macginleyi*
MIC	*Micrococcus* sp.

MIT	*Streptococcus mitis*
MNO	*Moraxella nonliquefaciens*
PAS	*S. pasteuri*
PRO	*Corynebacterium propinquum*
PSU	*Corynebacterium pseudodiptheriticum*
PYO	*Streptococcus pyogenes*
RAO	*Raoultella* sp.
SAP	*S. saprophyticus*
SCH	*S. schleiferi*
SCI	*S. sciuri*
SIM	*S. simulans*
SME	*Corynebacterium smegmentosum*
STR	*Streptococcus sp.*
WAR	*S. warneri*
XYL	*S. xylosus*

DNA	deoxyribonucleic acid
RDP	Ribosome Database Project
ABI	Applied Biosystems
dNTP	deoxyribonucleotide
agr	accessory gene regulator
AIC	Akaike Information Criterion
AIP	autoinducing peptide
ApoB	Apolipoprotein B
BHI	Brain Heart Infusion

CA-MRSA	Community acquired methicillin resistant *S. aureus*
ClfA	clumping factor A
ClfB	clumping factor B
ddH_2O	distilled water
DF	degrees of freedom
Eap	extracellular adherence protein
ESP	endoserine peptidase
FnbA	Fibronectin binding proten A
FnBP	fibronectin binding protein
GLM	generalised linear model
LRT	likelihood ratio test
MRSA	methicillin resistant *S. aureus*
OD	optical density
PBS	phosphate buffered saline
PC	principal component
PCA	principal component analysis
PCR	polymerase chain reaction
PIA	polysaccharide intercellular adhesion
Pls	Plasmin sensitive
PLV	Panton-Valentine leukocidin
PSM	phenol soluble modulin
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
Rot	repressor of toxins
rRNA	ribosomal ribonucleic acid

SasG	*S. aureus* surface protein G
SCIN	*Staphylococcus* complement inhibitor
SDS	sodium dodecyl sulphate
SNP	single nucleotide polymorphism
SSCP	Single strand conformation polymorphisms
TAE	Tris-acetate EDTA
TCSTS	two-component signal transduction system
UV	ultra violet

Units

°C	degrees celcius
cfu	colony forming units
g	grams
g L^{-1}	grams per litre
h	hours
kb	kilobase
kDa	kilodaltons
L	litres
M	Moles per Litre
mA	miliamps
mg	milgrams
min	minutes
ml	mililitre
mm	milimeters
ng	nanograms
nm	nanometers
RLU	relative light units
rpm	revolutions per minute
s	second
U	units
V	volts
v/v	volume per volume
w/v	weight per volume
µl	microlitre

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Abstract

The Ecology of *S. aureus*

Ben Libberton

Nasal carriage of *Staphylococcus aureus* is associated with increased risk of infection in humans. Several factors are known to affect carriage including host genetics and *S. aureus* immune evasion. However while members of the microbial community have been shown to affect *S. aureus* nasal colonisation individually, relatively little work has been done to understand how and why nasal microbial community as a whole, affects carriage of *S. aureus*. Here, the cultivable bacteria from 60 anterior nares communities were sampled and identified to species level using apiSTAPH and 16S rRNA gene sequencing. The taxa distributions across communities revealed negative associations *of S. aureus* with the following taxa: *S. capitis, Corynebacterium propinquum, C. macginleyi, Enterobacter aerogenes, S. epidermidis, Micrococcus* sp., *Bacillus* sp., *C. accolens, S. schleiferi* and *Gemella haemolysans*.

Since toxin-mediated interference can affect community composition, nasal isolates were screened for their ability to inhibit growth of *S. aureus* on a solid medium. Overlaying this inhibition data onto community taxa distributions revealed that negative associations between *S. aureus* and *S. epidermidis, S. capitis, C. propinquum, C. accolens* and a *Micrococcus* sp. were potentially driven by toxin-mediated interference competition. Moreover novel negative associates were found between *S. aureus* and an inhibitory subset of *Micrococcus luteus* and *S. hominis*. By also measuring the cumulative inhibition of entire natural communities, it was possible to show that *S. aureus* was less frequent in highly inhibitory microbial communities.

The quorum sensing mechanism, encoded by the *agr* locus, and biofilm formation have been proposed to play an important role in nasal colonisation of *S. aureus*. Therefore to further investigate community dynamics, *S. capitis, S. epidermidis* and corynebacteria isolates were assayed for their ability to interfere with Agr signaling and biofilm formation. No evidence was obtained to indicate that biofilm interference by these species affected the distribution of *S. aureus* across communities. By contrast, *S. epidermidis* isolates that interfered with Agr signaling were significantly more likely to coexist with *S. aureus*, and *S. capitis* isolates interfering with Agr signaling were significantly less likely to coexist with *S. aureus*.

In theory, toxin-mediated interference competition can act both to protect producers against invasion, and, conversely, to promote the invasion of producers into an occupied niche. An experimental ecology approach was used to show that *S. aureus* is less likely to invade an inhibitor-producing *S. epidermidis* population than a non-inhibitor-producing population, especially on a spatially-structured medium. Furthermore, inhibitor-producing populations of *S. epidermidis* invade more successfully than non-inhibitor-producers, although they do not displace the *S. aureus* resident due to evolution of toxin resistance. There is also evidence of co-evolution where inhibitor-producing strains of *S. epidermidis* can evolve stronger inhibitory activity when invading sensitive *S. aureus* populations that evolve resistance.

These findings could impact the future treatment of *S. aureus* infections and help to control nasal carriage.

Chapter 1: General Introduction

1.1 *Staphylococcus aureus* Carriage

Staphylococcus aureus colonises the anterior nares persistently in 20% of the human population and intermittently in a further 30%, with the remaining 50% being non carriers (van Belkum *et al.*, 2009). Higher carriage rates of *S. aureus* are associated with children (Peacock *et al.*, 2003), men (Cole *et al.*, 2001) and hospital patients (Goslings & Buchli, 1958). Moreover, carriage is most frequently associated with hospital patients with underlying health conditions including skin disease (Hoeger *et al.*, 1992), kidney dialysis (Yu *et al.*, 1986), or otherwise immunocompromised e.g. diabetes mellitus (Lipsky *et al.*, 1987) or HIV infection (Ho *et al.*, 2007). Social groups whose activities could facilitate *S. aureus* spread are also at risk of recurrent community-acquired Methicillin Resistant *S. aureus* (CA-MRSA) infection. Examples include: sports teams (Huijsdens *et al.*, 2006; Kazakova *et al.*, 2005; Nguyen *et al.*, 2005; Stacey *et al.*, 1998); military personnel (Beilman *et al.*, 2005; Ellis *et al.*, 2004; LaMar *et al.*, 2003; Zinderman *et al.*, 2004); children in daycare (Velazquez-Guadarrama *et al.*, 2009) and prison inmates (Gilbert *et al.*, 2006; Main *et al.*, 2005; Pan *et al.*, 2003).

Although nasal carriage is typically asymptomatic, carriers can act as a reservoir for endogenous opportunistic infection and have higher rates of disease (von Eiff *et al.*, 2001). These infections are often recurrent and respond poorly to treatment, even in immunocompetent hosts (Kreisel *et al.*, 2006). The risk of infection posed to immunocompromised carriers is

significantly higher, with increased severity and mortality rates (Hoen *et al.*, 1995; Senthilkumar *et al.*, 2001; Yu *et al.*, 1986). The spectrum of *S. aureus* infection is broad, ranging from relatively benign diseases such as superficial skin and soft tissue infections, like carbuncles and impetigo (Liu *et al.*, 2009; Raju *et al.*, 2010), to life-threatening infections such as endocarditis (Allue *et al.*, 2010; Giuliana *et al.*, 2010; Kern, 2010; Liu *et al.*, 2010) and pneumonia (Al-Talib *et al.*, 2011; Len *et al.*, 2010; Ott *et al.*, 2010). Frequently, life-threatening infections can progress from milder disease, thus rapid and effective treatment of complicated infections is critical. Over time the health threat has been exacerbated by the rapid evolution and spread of multi-antibiotic resistant clones which limits the efficacy of traditional therapies to which the bacteria have become resistant (Chua & Howden, 2009; Howden *et al.*, 2010; Lewis & Ellis, 2007; Moise *et al.*, 2009).

There are three main factors reported in the literature that govern carriage of *S. aureus*. These are firstly, host factors such as genetics (Foster, 2009; Sivaraman *et al.*, 2009) (discussed in section 1.1.1), secondly, factors associated with *S. aureus* and the way it alters gene expression to enhance its survival *in vivo* (Foster, 2009; Thoendel *et al.*, 2011) (section 1.1.2), and thirdly, the microbial community composition, which is the least well studied of the three and is the focus of this thesis (Frank *et al.*, 2010; Wos-Oxley *et al.*, 2010)(section 1.2).

1.2 Host factors

Host genetics determine many aspects of the nasal habitat and to some extent are known to determine *S. aureus* carriage. Perhaps unsurprisingly, host factors identified to affect carriage are typically associated with the host immune system. Polymorphisms in the glucocorticoid receptor gene alter the frequency of *S.aureus* carriage (van den Akker *et al.*, 2006). The glucocorticoid receptor is present on the surface of all human epithelial cells and, modulates transcription of anti-inflammatory genes in response to glucocorticoids. GG homozygotes of the exon 9β polymorphism were 68% less likely to be *S. aureus* carriers, whereas subjects possessing the codon 23 lysine allele were 80% more likely to be carriers (van den Akker *et al.*, 2006).

The exon 9β polymorphism causes an overactive immune response that is thought to also protect against *S. aureus.* The converse is true for subjects with the codon 23 lysine allele who have higher carriage rates. Moreover, SNP distribution in genes encoding C reactive protein and interleukin 4 are positively associated with *S. aureus* carriage (Ruimy *et al.*, 2010)

The human host also has mechanisms capable of interfering with the quorum sensing signalling in staphylococci called the agr system. The agr system allows individual staphylococcal cells to sense the density of cells around them possessing the same agr type, and regulate gene expression accordingly (discussed in greater detail in section 1.1.2). The agr system is inactivated by a pH lower than 5.5 (Regassa *et al.*, 1992) which can be found on the human skin and in the vaginal tract, which are relatively common

colonisation sites for *S. aureus* (Weinrick *et al.*, 2004). Agr has also shown to be inactivated by human serum (Yarwood *et al.*, 2002), which is not caused by low pH as human serum is neutral. Serum inhibition of agr is mediated by apolipoprotein B (ApoB), a component of human serum, which is essential for agr inhibition (Horswilll & Nauseef, 2008; Peterson *et al.*, 2008). It was suggested that ApoB can bind to native autoinducing peptides (AIPs) of all staphylococcal species and therefore sequesters them from use by the bacteria (Peterson *et al.*, 2008). Haemoglobin is another blood component with agr interference properties, although the exact nature of them is unclear. The α and β chains of the haemoglobin molecule were able to repress RNAIII, possibly through an electrostatic interaction with the surface receptor AgrC (Schlievert *et al.*, 2007).

1.3 *S. aureus* factors

1.3.1 Immune Evasion

A potential factor that might contribute to *S. aureus* nasal colonisation is its ability to evade the host immune response. The human host has a complex immune system with many mechanisms for killing bacteria that elicit an immune response. The complement cascade is an important arm of the innate immune system and responsible for directly killing bacterial cells and targeting cells for phagocytosis (Ben Nasr *et al.*, 2006; Payne & Horwitz, 1987; Schlesinger *et al.*, 1990). Complement consists of around 25 different proteins (Stoermer & Morrison, 2011) that are recruited to either facilitate bacterial killing by destroying the membrane potential of Gram-negative bacterial cells via the membrane attack complex, or via opsonisation, the

marking of cells for phagocytosis. *S. aureus* is able to inactivate the complement cascade by secreting a 9.8-kDa protein called the *Staphylococcus* complement inhibitor (SCIN) (Rooijakkers *et al.*, 2006). Secretion of SCIN prevents complement activation and blocks subsequent recruitment of phagocytes. The *S. aureus* secreted fibrinogen-binding protein Efb was shown to bind the complement component C3 and prevent it from opsonising the cell (Lee *et al.*, 2004). Even if complement factors such as C3b and IgG are allowed to bind to the bacterial cell surface, *S. aureus* is able to cleave both of these molecules by secreting staphylokinase which activates plasminogen, the active form of which (plasmin) is able to cleave both C3b and IgG (Rooijakkers *et al.*, 2005).

In addition, *S. aureus* also produces a number of cell surface molecules that prevent cells being opsonised. One such molecule is protein A, which is a surface anchored protein. It has two domains that are capable of binding the Fc portion of IgG. (Deisenhofer, 1981; Uhlen *et al.*, 1984). Binding the Fc region puts the IgG in the wrong orientation to mark the *S. aureus* cells for phagocytosis. *In vitro* studies have shown that *S. aureus* mutants deficient in protein A are phagocytosed more readily by neutrophils (Gemmell *et al.*, 1991) and are less virulent in animal models (Palmqvist *et al.*, 2002; Patel *et al.*, 1987). Another important antiphagocytic cell surface component is clumping factor A (ClfA). ClfA is a cell surface protein that is upregulated in post exponential phase and is present on the cell surface in stationary phase (Bischoff *et al.*, 2004; O'Brien *et al.*, 2002b). ClfA is a fibrinogen binding protein that, when expressed *in vivo,* results in the cell being coated with

fibrinogen, thereby preventing deposition of opsonins on the cell surface. Indeed studies show that ClfA increases virulence in a murine model (Josefsson *et al.*, 2001) and inhibits phagocytosis by macrophages (Palmqvist *et al.*, 2004). Many clinical isolates of *S. aureus* also possess a polysaccharide capsule (O'Riordan & Lee, 2004; Roghmann *et al.*, 2005). While these capsules do not prevent complement proteins from binding to the cell surface by assembling under the capsule, bacteria are not phagocytosed, indicating that the complement proteins may not be accessible to the neutrophils (Nilsson *et al.*, 1997; Thakker *et al.*, 1998).

S. aureus can indirectly avoid phagocytosis by inhibiting neutrophil chemotaxis. Many *S. aureus* strains produce chemotaxis inhibitory protein of staphylococci (CHIPS). CHIPS can bind to the neutrophil receptors (formyl peptide receptor and C5aR receptor) and prevent binding of the chemo-attractant signal produced at the site of an *S. aureus* infection (de Haas *et al.*, 2004; Veldkamp *et al.*, 2000). A second protein, the extracellular adherence protein (Eap), competitively binds to the ICAM-1 receptor on the surface of neutrophils, excluding the lymphocyte-function-associated antigen that also stimulates recruitment of the neutrophil to the site of infection (Chavakis *et al.*, 2002).

S. aureus is also capable of killing leukocytes via the secretion of pore forming exotoxins. The Panton-Valentine leukocidin (PLV) is the most well known and leukocidin that *S. aureus* strains posess and is associated with extremely high virulence (Colin *et al.*, 1994; Genestier *et al.*, 2005).

S. aureus is resistant to many antimicrobial molecules produced by the human host. Lysozyme is an important enzyme in the innate immune system. It is a muramidase, and breaks down the bacterial cell wall by cleaving the 1-4 glycosidic bond between the N-acetylglucosamine and the N-acetyl muramic acid. The enzyme is present in many body fluids such as nasal secretions and tears (Cole *et al.*, 1999), however, *S. aureus* cells secrete a membrane bound O-acetyl transferase that acetylates the N-acetyl muramic acid, which together with teichoic acid, prevents access of lysozyme to the glycosidic bond (Bera *et al.*, 2005). Furthermore, *S.aureus* is resistant to many human antimicrobial peptides (Peschel & Collins, 2001; Peschel *et al.*, 2001; Peschel & Sahl, 2006). These cationic molecules are attracted to the anionic bacterial cell surface, where they integrate into and disrupt the lipid bilayer. *S. aureus* possesses at least two mechanisms that reduce negative charge on the cell surface to repel these cationic antimicrobial peptides. Dlt, catalyses D-alanine substitutions in lipoteichoic acid and ribitol teichoic acid in the cell membrane (Peschel *et al.*, 1999). MprF adds L-Lysine to the phosphatidylglycerol present on the cell surface (Peschel *et al.*, 2001; Staubitz *et al.*, 2004). Both *mprF* and *dlt* operon mutants are more susceptible to cationic antimicrobial peptides *in vitro* and have reduced virulence in an animal model (Collins *et al.*, 2002; Kristian *et al.*, 2003). Resistance to antimicrobial peptides allows *S. aureus* to survive inside the phagosome of professional phagocytes (Kubica *et al.*, 2008). Antimicrobial peptides are also secreted into the nares by the nasal epithelium (Laubel *et al.*, 2006).

Staphyloxanthin, the orange carotenoid pigment in the *S. aureus* membrane, protects against the oxidising effects of free radicals present in the phagosome (Liu *et al.*, 2005). Moreover, *S. aureus* has two superoxide dismutase enzymes detoxify reactive oxygen species (Karavolos *et al.*, 2003). The presence of divalent manganese in the bacterial cell can also remove reactive oxygen species; mutants deficient in manganese uptake were shown to be less virulent in a murine model (Horsburgh et al., 2002). Another way in which reactive oxygen interrupts cellular processes is by oxidising the sulphur atom of the amino acid methionine. *S. aureus* circumvents this by producing three different methionine sulphoxide reductases (Singh & Moskovitz, 2003) which are important for virulence in mice (Mei *et al.*, 1997).

1.3.2 S. aureus adherence to host factors

Many bacterial proteins have been identified as important for colonisation. The majority of these proteins, understandably, have a role in adherence of the staphylococcal cell to human extracellular matrix components. Several *S. aureus* proteins are known to interact with fibrinogen, which is a component of the human extracellular matrix. IsdA (Clarke *et al.*, 2004), Fibronectin binding proten A (FnbA) (Heilmann *et al.*, 2004), extracellular adherence protein (Eap) (Kreikemeyer *et al.*, 2002) and clumping factor B (ClfB) (O'Brien *et al.*, 2002b), have all been shown to enhance colonisation by binding to fibronectin. Eap is also thought to link to and facilitate adherence via other *S. aureus* adherence factors (Hussain *et al.*, 2002). ClfB has also

been shown to bind cytokeratin 10, which is present on the surface of all desquamated nasal epithelial cells, as well as human keratinocytes (O'Brien *et al.*, 2002b). SdrE (extracellular matrix binding protein) is predicted to have a similar structure to ClfB and is also important for host cell attachment (O'Brien *et al.*, 2002a). Two further *S. aureus* proteins, SasG (*S. aureus* surface protein G) and Pls (Plasmin sensitive) also bind to cytokeratin 10 and enhance adherence to epithelial cells (Roche *et al.*, 2003).

1.3.3 The S. aureus quorum sensing system and factors under its control

Quorum sensing is a mechanism by which microorganisms can synchronously sense and alter gene expression within an entire population in response to cell density. Signalling occurs via secretion of diffusible molecules, which either effect gene expression directly or by means of a two-component signal transduction system (TCSTS). The quorum sensing TCSTS in *S. aureus* is called agr. In *S. aureus*, a range of genes proposed to be important to nasal colonization are under agr regulation, therefore the agr system in *S. aureus* will be briefly described before going on to describe specific gene products. The quorum sensing TCSTS in staphylococci is the *agr* system (Fig. 1.2). The divergent *agr* operons are transcribed from two promoters P2 and P3. P2 regulates the transcription of the TCSTS and the signalling molecule AIP (Janzon & Arvidson, 1990; Novick *et al.*, 1993). The TCSTS is comprised of the receptor histidine kinase AgrC, and AgrA, the response regulator. P3 regulates synthesis of RNAIII, a transcription and translation regulator (riboregulator) for all the genes associated with the agr system except the response regulator AgrA. The function of AgrA seems only

to be to upregulate transcription at both promoters P2 and P3 to produce more of the TCSTS and RNAIII (Novick *et al.*, 1993; Novick *et al.*, 1995).

The auto-inducing peptide (AIP) of the staphylococci is a derivative of AgrD, which has both the N- and C-terminus processed by AgrB (Lyon & Novick, 2004). While the N-terminal processing appears only to be proteolytic cleavage (Lyon & Novick, 2004), the processing at the peptide's C–terminus results in condensation and cleavage, creating a thioester link between the C-terminus and a cysteine residue, which is conserved in most AgrD genes (Kalkum *et al.*, 2003; Nakayama *et al.*, 2001b). This linkage produces a thiolactone ring at the C–terminus and gives the staphylococcal AIP its characteristic shape (Fig. 1.2), which is critical for its function as a signalling peptide (Ji *et al.*, 1997).

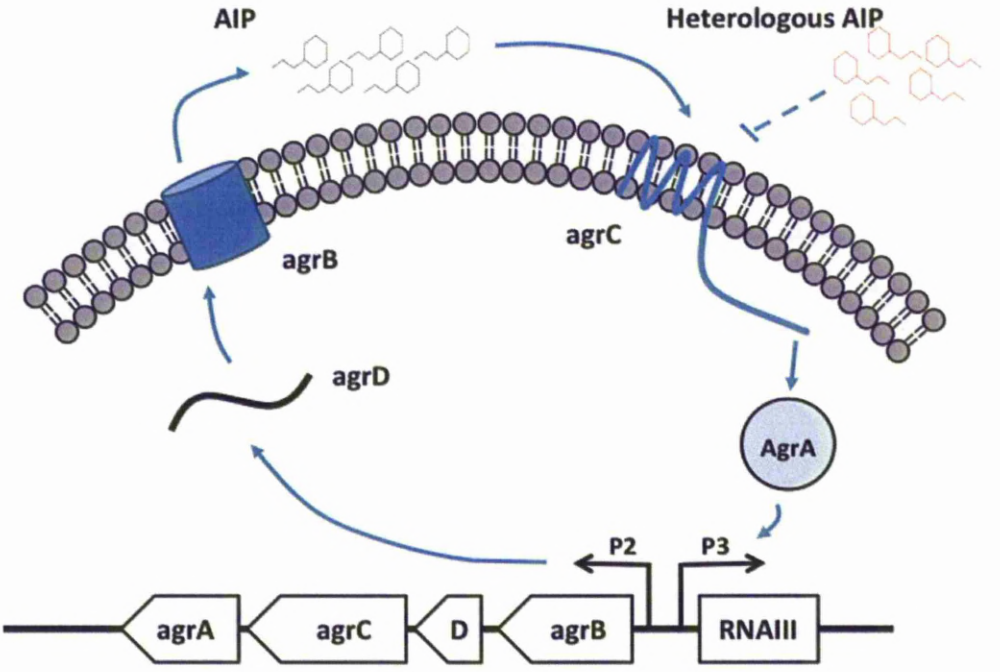


Figure 1.1. Schematic of the genes and gene products of the agr system. Adapted from (Novick & Geisinger, 2008). The grey circles represent the phospholipid bilayer. Below is the cytoplasmic region and above is outside the bacterial cell.

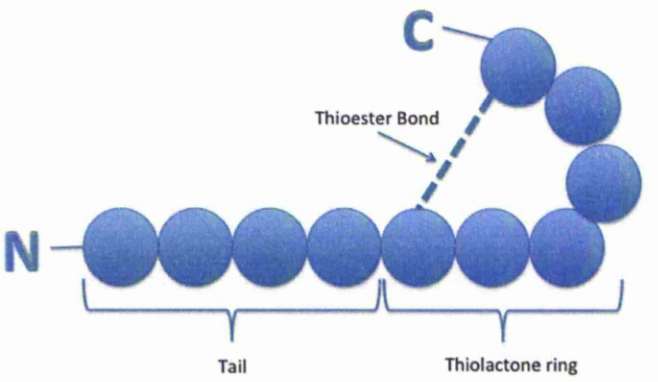


Figure 1.2 AIP structure. Each circle represents a different amino acid residue. The N- and C- termini are marked by N and C. The distinctive shape is derived from the thiolactone ring that binds to the AgrC receptor and the tail, which is responsible for signal transduction.

The agr regulon contains a diverse array of genes. Those upregulated by the agr system are expressed in late exponential phase and some are associated with virulence (Thoendel *et al.*, 2011). These genes include secreted enzymes e.g. proteases and lipases (Kalkum *et al.*, 2003; Oscarsson *et al.*, 2006; Shaw *et al.*, 2004), toxins such as haemolysins, enterotoxins and superantigens (Recsei *et al.*, 1986; Regassa *et al.*, 1991) and immunomodulatory peptides such as phenol soluble modulins (αPSMs & βPSMs) (Queck *et al.*, 2008). Few genes are down regulated by agr (Thoendel *et al.*, 2011), but the majority that are encode surface proteins e.g. protein A (Huntzinger *et al.*, 2005) and fibronectin binding proteins (Saravia-

Otten *et al.*, 1997). The agr system also downregulates the Rot (repressor of toxins) transcription factor, further increasing toxin production (Boisset *et al.*, 2007).

RNAIII is the main effector in the agr system; it affects gene expression at the transcriptional level, but also and most commonly at the post-transcriptional level by stabilising or destabilising mRNA and inhibiting its translation (Boisset *et al.*, 2007; Huntzinger *et al.*, 2005). RNAIII is 514 nucleotides long and has 14 hairpin loops, making it one of the most complex RNA regulators found in the bacterial kingdom (Benito *et al.*, 2000). Most of the transcriptional regulation of RNAIII is mediated through the repression of Rot (Geisinger *et al.*, 2006). RNAIII binds at the Shine-Dalgarno sequence of the *rot* mRNA, thereby preventing translation of the repressor of most of the *S. aureus* toxins (Boisset *et al.*, 2007). RNAIII also recruits RNAseIII, which cleaves double stranded RNA, causing cleavage of the *rot* Shine-Dalgarno sequence where it is bound to RNAIII, significantly reducing the half-life of the *rot* mRNA transcript (Boisset *et al.*, 2007).

RNAIII can also promote translation of mRNA transcripts by binding to a hairpin loop in mRNA thereby liberating the Shine-Dalgarno sequence to be recognised by the 16S ribosomal subunit. An example of this regulatory control is observed via RNAIII promoting the translation of the α-haemolysin gene *hla* (Morfeldt *et al.*, 1995; Novick *et al.*, 1993). This mechanism is also an example of the complexity of the regulation in this system since *hla* is

regulated at the level of transcription by Rot (Cheung *et al.*, 1992; Giraudo *et al.*, 1997; Said-Salim *et al.*, 2003)

1.3.4 Do biofilms play a role in S. aureus nasal colonization?

S. aureus biofilm formation is proposed to contribute to pathogenesis for many chronic diseases, such as endocarditis (Frank *et al.*, 2008; Parsek & Greenberg, 2005), osteomyelitis (Brady *et al.*, 2008; Costerton, 2005) and infections associated with indwelling medical devices (Otto, 2009; Rogers *et al.*, 2009). However, it is unknown if *S. aureus* form biofilms during nasal colonisation (Krismer & Peschel, 2011). Disruption of a *S. aureus* biofilm by *S. epidermidis* was attributed to displacement of *S. aureus* from the nose, supporting a role in colonisation (Iwase *et al.*, 2010). Furthermore, the ability of *S. aureus* to colonise the nasal epithelium was associated with an ability to form *in vitro* biofilms (Quinn *et al.*, 2009). However, histological studies of cotton rats and humans failed to show signs of biofilm formation during *S. aureus* nasal colonisation (Nouwen *et al.*, 2004b; ten Broeke-Smits *et al.*, 2010). *S. aureus* biofilm formation therefore potentially represents an important field of investigation with respect to nasal carriage. *S. aureus* biofilms are classed either as polysaccharide intercellular adhesion (PIA) dependent or PIA independent. In the latter case biofilms are mediated by protein interactions, which can be disrupted by low specificity proteases (trypsin and proteinase K) (Beenken *et al.*, 2003; Beenken *et al.*, 2010; Lauderdale *et al.*, 2009). Agr expression reduces biofilm formation (Beenken *et al.*, 2003; Vuong *et al.*, 2000). This may be because agr downregulates cell surface adhesins (Huntzinger *et al.*, 2005; Saravia-Otten *et al.*, 1997).

However, a more credible mechanism is that agr upregulates the production of many different secreted proteases (Oscarsson *et al.*, 2006; Shaw *et al.*, 2004) which then disrupt PIA-independent biofilms (Boles *et al.*, 2010; Marti *et al.*, 2010; O'Neill *et al.*, 2007; Tsang *et al.*, 2008). The targets of these proteases are unknown. Nevertheless, candidates include the fibronectin binding proteins (FnBPs), which are known to be important for biofilm formation in *S. aureus* (O'Neill *et al.*, 2007). Agr not only downregulates FnBPs (Saravia-Otten *et al.*, 1997) but it also upregulates *SspA* (the V8 protease) which was shown to cleave FnBPs (McGavin *et al.*, 1997). Furthermore, agr upregulates the production of Phenol Soluble Modulins (PMSs) (Queck *et al.*, 2008); these are amphipathic molecules with surfactant properties that are capable of dispersing a biofilm (Boles *et al.*, 2005; Davey *et al.*, 2003; Irie *et al.*, 2005). These various modes of biofilm disruption are thought to be responsible for the promotion of systemic *S. aureus* infections and transmission resulting from a single biofilm (Fux *et al.*, 2004).

1.4 Microbial community factors

Compared to the numerous studies investigating host and *S. aureus* specific factors affecting nasal carriage, relatively few studies have investigated the contribution of the microbial communities. There is growing evidence that microbial community interactions can determine the presence of a particular species in a community (Margolis & Levin, 2007; Pham *et al.*, 2005; Taga, 2007; Tong *et al.*, 2007; Uroz *et al.*, 2005). Indeed, several studies have identified interactions between species within nasal communities that

potentially contribute to the distribution of *S. aureus* across communities (Frank *et al.*, 2010; Lina *et al.*, 2003; Wos-Oxley *et al.*, 2010). Before detailing specific, known interactions, some community ecology concepts will briefly be introduced.

A community can be defined as an assemblage of populations of two or more species that interact in a defined site (Morin, 1999). Community composition at a given site is thought to be affected by several factors (Fig. 1.3), these include: dispersal limitation - whereby only a subset of possible species present in a regional species pool can access a given site; environmental constraints – whereby only a subset of species can persist under the environmental conditions prevailing at a given site; species interactions – whereby ecological interactions between species determine the subset capable of coexistence (Fig. 1.3).

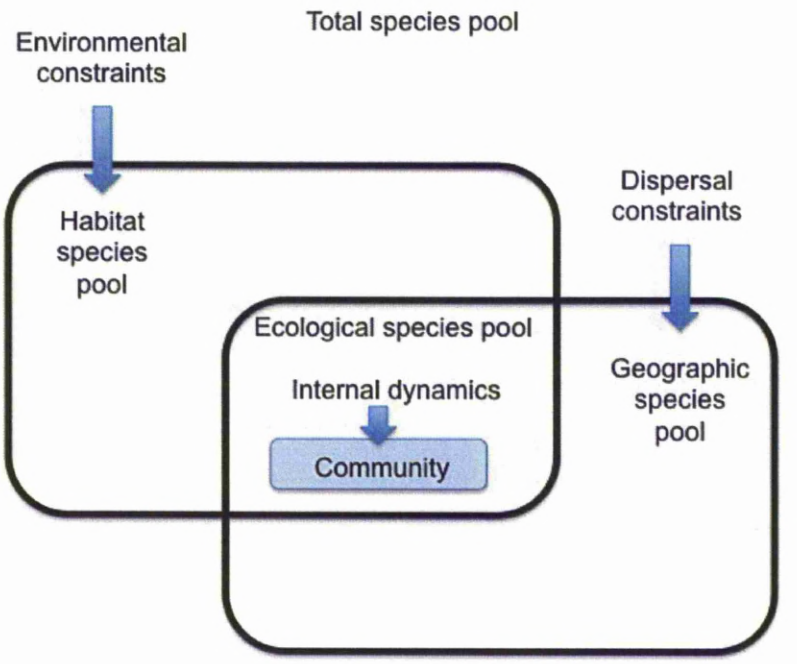


Figure 1.3. Factors affecting community composition. There are five different species pools: the total species in a region, the habitat species pool (species able to survive at a site), the geographic species pool (species physically able to arrive at a site), the ecological species pool (species that can both arrive at the site and persist) and the community which are drawn from the ecological pool and can survive internal biotic dynamics. Adapted from Begon *et al.* (1997).

An important first step in characterising a community is measurement of the distribution and abundance of species (Morin, 1999). Diversity can be measured over a range of spatial scales. Alpha diversity, measures the diversity of individual local communities, and can be quantified as simple species richness – the total number of species present in a given measured area or community – or using a diversity index, such as the Simpson index and the Shannon Weaver index, which take into account the species richness and the proportional abundance of each species in a specific locality

(Shannon & Weaver, 1964; Simpson, 1949). Gamma diversity measures regional diversity (Whittaker, 1972) such that it can be thought of as the combined alpha diversities of a number of local communities, often collectively referred to as the metacommunity (Wilson, 1992). Alpha and gamma diversity are linked by beta diversity, a third measure that describes the dissimilarity between the composition of pairs of local communities (Fig. 1.4) (Whittaker, 1972). Beta diversity indices include the Bray-Curtis dissimilarity (Bray & Curtis, 1957) and the Whittaker index (Whittaker, 1960). Low beta diversity indicates that local communities are homogeneous whereas high beta diversity shows local communities are highly heterogeneous. These quantitative aspects of diversity are elaborated on in the introduction to chapter 3.

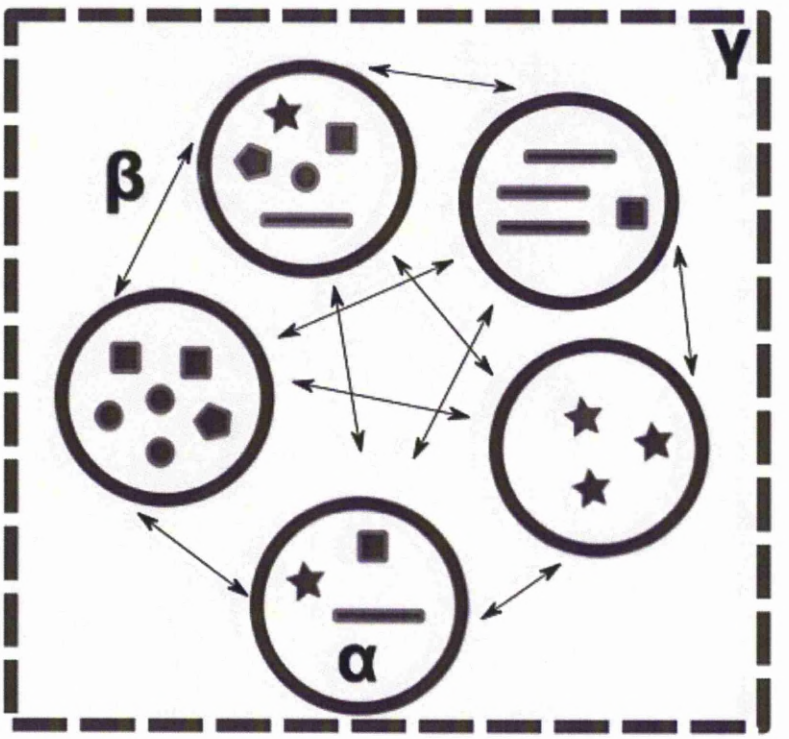


Figure 1.4. Alpha, Beta and Gamma diversity perameters as described by Whittaker (1972). Gamma (γ) diversity is represented by the dotted line surrounding all of the local communities (large circles). The diversity within local communities (large circles), i.e. the number of different shapes is designated alpha (α) diversity. The difference between pairs of local communities (double arrow) represent beta (β) diversity.

Microbes inhabiting the anterior nares potentially influence the presence, distribution and abundance of *S. aureus*. These effects might occur through occupation of space within the nasal niche, interactions between co-colonising microbial species or secretion of antimicrobial molecules. To investigate microbial influences on carriage of *S. aureus* the composition of nasal communities associated with different individuals must first be characterised. By using diversity indices to describe the nasal microbial communities, it is possible to make comparisons between them. By measuring the similarity and dissimilarity e.g. beta diversity, it is possible to infer interactions between community members. Negative presence/absence associations between species, which cause high beta diversity, imply mutual exclusion, while positive associations between species' distributions imply potential niche differentiation or facilitation allowing coexistence (Frank *et al.*, 2010; Wos-Oxley *et al.*, 2010).

1.4.1 Inferred interactions between members of the nasal microbial community

Previous surveys have sampled the nasal microbial communities to look specifically at carriage of *S. aureus* in a variety of settings (e.g., hospitals (Marques *et al.*, 2010; Motamedifar *et al.*, 2010; Shakya *et al.*, 2010; Shrestha *et al.*, 2009), student populations (Prates *et al.*, 2010), children (Oguzkaya-Artan *et al.*, 2008) microbiology laboratories (Jager *et al.*, 2010), however few have attempted to identify co-colonising microbes. Two quantitative culture dependent studies have been performed to assess the entire nasal microbial community (Heczko *et al.*, 1981; Rasmussen *et al.*,

2000). (Heczko *et al.*, 1981) examined the aerobic and anaerobic microbial populations of eight subjects. Despite being a small survey, Heczko *et. al.* (1981) reported that *S. aureus'* presence was associated with diminished numbers of several species of staphylococci (*S. epidermidis, S. xylosus and S. hominis*) and propionibacteria (*P. acnes, P. granulosum* and *P. avidum*) suggesting these species can possibly exclude *S. aureus* from the niche. A second culture-dependent study (Rasmussen *et al.*, 2000) focused on the aerobic microbiota of 10 healthy human subjects. This study compared the microbial communities from different sites within the nasal cavity rather than compare community composition between noses. *S. aureus* was only isolated from one of the volunteers and represented only 0.01%, of all the bacterial cells sampled. As such no conclusions could be drawn regarding the effect of the microbial community on *S. aureus* carriage. Recently, and after this study commenced, culture-independent approaches were used as powerful tools to study microbial communities. Single strand conformation polymorphisms (SSCP's) combined with 454- pyrosequencing were used to analyse 16S rRNA gene diversity in samples from the noses of 40 volunteers (Wos-Oxley *et al.*, 2010). This study reported negative associations between *S. aureus* and *Finegoldia magna*, and *S. aureus* and *Corynebacterium accolens*. By contrast, *C. pseudodiptheriticum* was strongly positively associated with *S. aureus*. Another recent culture-independent study using 16S rRNA gene sequencing (Frank *et al.*, 2010) compared the nasal microbial communities of healthy individuals and hospital inpatients. Hospitalised patients were enriched for *S. aureus* or *S. epidermidis* and lacking *P. acnes*, similar to the culture dependent findings described by

Heczko *et. al.* (1981) nearly three decades previously. Furthermore, Frank, *et. al.* (2010) reported a significant negative association between *S. aureus* and *S. epidermidis* as shown previously by Lina, *et. al.* (2003). This putative negative association was subsequently confirmed by inoculating human *S. aureus* carriers with *S. epidermidis* strains. *S. aureus* was completely displaced by *S. epidermidis* strains possessing the *esp* gene, which encodes a protease that was shown to disrupt a *S. aureus* biofilm (Iwase *et al.*, 2010).

1.4.2 Types of interspecific competition

Negatively associated species distributions suggest competitive interactions between species that prevent coexistence. Several types of interspecific competition could potentially drive negative associations between *S. aureus* and other nasal community members.

1.4.3 Preemption

Preemptive competition describes when one species occupies the space in a niche first so there is physically no colonization space for other species. Unless an invading species can displace the established species, then the resident species will outcompete the invader. In this case species with high dispersal rates are often highly competitive as they can occupy a larger proportion of the available habitats and exclude competitors simple by occupying space. This was shown to occur with the microbial communities in the human gut (Fujiwara *et al.*, 1999; Gopal *et al.*, 2001). Preemptive competition has not been shown explicitly for *S. aureus* but binding to nasal epithelial cells is important for colonisation (O'Brien *et al.*, 2002b). It is

reasonable to speculate therefore, that if a competitor occupies binding sites on the nasal epithelium, *S. aureus* colonisation would be restricted.

1.4.4 Resource competition

Resource competition occurs when two or more species in a community are competing for a shared resource. As one species consumes a finite resource there is less available for the other species requiring this resource that will suffer as a result. Logically, species acting in their own interest will consume a limiting resource until it is depleted, resulting in a population crash known as the tragedy of the commons (Hardin, 1968). Tragedies of the commons can be prevented by competitors using resources more slowly (Kreft, 2004), or by using a different resource, a concept known as niche partitioning (Barker *et al.*, 1997). Iron scavenging resource competition between *S. aureus* and *Ps. aeruginosa* has been identified *in vitro* (Harrison *et al.*, 2008) and *in vivo* by an rat infection model (Mashburn *et al.*, 2005). However, this has not yet been demonstrated in the nasal environment.

1.4.5 Toxin mediated interference

Toxin mediated interference competition is caused by the production of a compound by one organism that reduces the relative fitness of another organism in the community (Morin, 1999). Toxin mediated interference is common in bacteria from the production of bacteriocins (Jack *et al.*, 1995), antibiotics (Rasool & Wimpenny, 1982) and secondary metabolites (Leão *et al.*, 2009; Tagg *et al.*, 1976).

However toxin mediated interference has not been described in relation to the nasal microbial community or to colonisation. This is despite the fact that the staphylococci possess a wide range of different bacteriocins capable of killing closely related species (Hechard & Sahl, 2002; Netz *et al.*, 2001; Peschel *et al.*, 1997; Schnell *et al.*, 1988). Two bacteriocins produced by *S. epidermidis* have been well characterised and display killing activity against *S. aureus;* these are epidermin (Fontana *et al.*, 2006) and gallidermin (Peschel *et al.*, 2001).

1.4.6 Agr interference drives competitive interactions in the niche

Inhibition of agr signalling can significantly reduce *S.aureus* fitness. The agr system is universally present throughout the staphylococci and similar systems have also been found in other closely related genera. Interestingly, many studies have shown heterologous AIPs are capable of inhibiting agr signalling by binding the AgrC receptor from a different agr system. *S. aureus* possesses four different agr systems all with AIPs capable of inhibiting one another (Ji *et al.*, 1997). The interference comes from the fact that each of the AIPs can still bind to the AgrC receptor, but lack specificity in key amino acids to transduce a response to the sensor histidine kinase. AIP-II was shown to have a particularly inhibitory effect against the other three *S. aureus* agr types, as well as attenuating their virulence in a murine model (Ji *et al.*, 1997; Mayville *et al.*, 1999). Fleming *et al* (2006) also showed that competition between different agr types in an insect model also caused reduced virulence. Most AIP peptides with N-terminal truncations were also shown to strongly interfere with agr signalling (Lyon *et al.*, 2000), resulting

from competitive and irreversible binding of the truncated AIP to the AgrC active site, hence blocking it from native AIP of the same agr type.

It must also be noted that the agr phenotype is by no means consistent across staphylococcal strains. For example, *S. aureus* strains MRSA252 and USA400 both posess the agr-III system, although the agr signal produced by MRSA252 is weak (Cassat *et al.*, 2006) whereas the USA400 strains have a relatively strong agr system (Montgomery *et al.*, 2008). This factor adds an extra layer of complexity to the interference competition observed between agr types, as the strength, i.e. the quantity of AIP that is produced has a strong bearing on the outcome of interference competition. This is especially true when considering competition involves competitive binding of the AIP to the AgrC molecule, and the concentration of competing AIP will have a direct impact on the interaction.

S. epidermidis contains heterologous AIPs which are capable of inhibiting agr signalling in *S.aureus* to varying degrees. Lina *et al.* (2003) confirmed that different *S. aureus* AIP are rarely isolated from the same niche, but also that *S. epidermidis* AIPs 1, 2 and 3 are significantly less likely to coexist with *S.aureus* AIP 2, and *S. epidermidis* AIP 3 is also unlikely to coexist with *S. aureus* AIP 1. This study also revealed that only one *S. aureus* agr type was ever isolated from a sampled niche, implying that agr competiton might influence colonisation.

1.4.7 Biofilm disruption

S. epidermidis strains that secreted an endoserine peptidase, (Esp) were shown to disrupt *S. aureus* biofilms *in vivo.* When introduced into the anterior nares, the Esp producing strains displaced *S. aureus* from the noses of human carriers, suggesting that this mechanism might function in the natural environment (Iwase *et al.*, 2010). However, this Esp study did not address *agr*-interference as described by Lina *et al.* (2003). A similar challenge study showed that a wild-type *Corynebacterium* sp. could displace *S. aureus* from the nares of persistent carriers, although the mechanism was not identified (Uehara *et al.*, 2000).

1.4.8 Competition mediated by phage induction

An inverse correlation between *S. aureus* and *Strep. pneumoniae* colonisation in infants has been described (Bogaert *et al.*, 2004). This is attributed to vaccinations against *Strep pneumoniae* that lead to a higher incidence of *S. aureus* otitis media in children, implying *Strep. pneumoniae* was competing with *S. aureus* and limiting infection (Bogaert *et al.*, 2004). A potential mechanism derives from the fact that *Strep. pneumoniae* produces hydrogen peroxide which, while not produced at high enough concentrations in the niche to kill *S. aureus,* is sufficient to induce the stress response to induce the lytic cycle of temperate phage (Selva *et al.*, 2009).

1.5 Aims

The overall aims of this thesis were as follows:

[1] To characterise the structure of the microbial metacommunity inhabiting the human anterior nares

[2] To determine the contribution of species interactions to the distribution of *S. aureus* across communities

[3] To identify species capable of excluding or preventing colonisation by *S. aureus* and to begin to elucidate the mechanisms of such inhibition

Chapter 2: Materials and Methods

2.1 Bacterial Growth conditions

Bacteria were routinely cultured in 10 ml Brain Heart Infusion (BHI) broth (LabM) in a 20 ml glass universal tube. Cultures were grown at 37°C, shaken at 200 rpm. Overnight cultures were typically grown for around 18 h. Exact details of the media can be found in Table 2.1. Strain stocks were maintained by adding either 700 µl of an overnight culture, or a single colony picked from a plate and resuspended in 700 µl of BHI broth (LabM) to 300 µl of 50% (v/v) glycerol and freezing at -80°C. Duplicates were made of all freezer stocks.

2.2 Genomic DNA extraction

DNA was extracted from a 1.5 ml aliquot of a 10 ml overnight culture. The cells in the 1.5 ml aliquot were harvested by centrifugation at 1400 rpm and resuspended in lysis buffer (Table 2.1) supplemented with 20 mg ml^{-1} lysozyme and 20 mg ml^{-1} lysostaphin, just before use. The Gram-positive organism genomic DNA extraction method was then used as described in the manufacturer's instructions for the DNeasy Blood and Tissue Kit (Qiagen).

2.3 Polymerase Chain Reaction

A recombinant, non-proof reading, Taq DNA polymerase (Bioline) was routinely used to amplify DNA. For more stringent DNA amplification 2.5 U of ACCUZYME™ DNA Polymerase (Bioline) was added. Typical thermocycling conditions comprised of an initial denaturation of 5 min at 95°C, 30 cycles of denaturation at 95°C for 60 s, annealing for 1 min [temperature determined

by specific primers], extension at 72°C for 30 s per kb of product, followed by a final extension of 72°C for 7 min. All PCR amplifications were carried out on one of the following thermocyclers: G-Storm GS1 (GRI), MJ research DNA engines (Bio-Rad) or Techne TC-312 (Techne). Primers specific to each experiment were synthesized by Sigma-Aldrich. Templates for the PCR (50 µl reaction) were 1-2 µl of DNA preparation (1-100 ng). A negative control comprising of ddH_2O in place of the template was always included.

2.4 Agarose Gel Electrophoresis

DNA was separated by electrophoresis in a 1% (w/v) agarose gel with TAE buffer at 100 V, unless otherwise stated. Hyperladder I (Bioline) was used as the DNA size marker. The gels were visualised under a UV transilluminator at a wavelength of 302 nm and images were recorded using GeneSnap software (Syngene).

2.5 Identification of 16S rRNA gene sequences

Presumptive staphylococcal isolates were identified using API staph (BioMérieux, Marcy-l'Etoile, France). For all other isolates, and where API STAPH failed to provide an identity, 16S ribosomal DNA sequencing was used for identification. Genomic DNA was extracted from a 1.5 ml aliquot of a 10 ml overnight culture. The cells in the 1.5 ml aliquot were harvested by centrifugation and resuspended in lysis buffer supplemented with 20 mg ml^{-1} lysozyme and 20 mg ml^{-1} lysostaphin, specficially for Gram-positive organisms before genomic DNA extraction using a DNeasy Blood and Tissue Kit (Qiagen). The 16S rRNA gene was amplified using primers pA and pH

(Table 2.2) (Edwards *et al.*, 1989) with BioMix Red polymerase (Bioline) supplemented with 2.5 U ACCUZYME™DNA Polymerase (Bioline) according to manufacturer's instructions using the following PCR cycling conditions: denaturation at 95°C for 5 min, followed by 30 cycles [95°C for 1 min, 55°C for 1 min, 72°C for 1.5 min] and a final extension at 72°C for 7 min. The PCR products were checked for purity by electrophoretic separation on a 1% (w/v) TAE agarose gel (agarose from Bioline) run in TAE buffer for 45 minutes at 80 V. A 30 µl aliquot of the PCR reaction was incubated with ExoSAP-IT® (USB,Staufen, Germany) (method 2.11) to dephosphorylate primers and dNTPs according to the manufacturer's instructions. The treated PCR product was then sequenced using the forward pA primer by GATC (Konstanz, Germany) using the Sanger method on an ABI 3730xl sequencing machine. The 16S rRNA gene sequences were analysed with the Sequence Match program on the RDP website (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) (Michigan State University) to identify the closest matching sequences. Bacterial species identities were assigned based on the matching sequence with the lowest e-value, i.e. the lowest chance of a match occurring by chance, accounting for the quality and length of the sequence, as well as the size of the database. Each identity was assigned only if there were no other identities in the database with the same e-value. Any sequence identities with the same e-value were re-sequenced.

2.6 Inhibition Spray Assay

The inhibition spray assay was based on the protocol described previously by Nascimento et al. (2006), using SH1000 as the indicator strain. A 25 µl spot (approximately 10^8 cells) of an overnight bacterial culture (method 2.1) of each nasal isolate was pipetted onto the centre of an agar plate containing 15 ml of BHI agar (lab M). The plates were incubated for 18 h at 37°C before 250 µl of a ten-fold diluted overnight culture of *S. aureus* SH1000 (10^6 cfu) was sprayed over the plate. The plates were incubated for a further 18 h after when the size of the inhibition zone produced by the central nasal isolate on SH1000 was assessed. The clarity of the inhibition zone was scored based on a simple scoring system of 1 to 4, 4 being completely clear and 1 being no detectable zone. The areas of any detectable zones were also recorded by measuring the diameter of the inhibition zone and the central colony. The area of both the zone and the colony were calculated using the equation $A = \pi(d/2)^2$ where d is the diameter of the colony or the inhibition zone. The central colony area was then subtracted from the total zone area, leaving only the area of the zone around the perimeter of the central colony.

2.7 Urease Assay

1 ml of urease reagent (Table 2.1) was sterilized in a 1.5 ml eppendorf. A single colony of the test organism was added to the eppendorf and incubated until the positive control organism (*Proteus mirabilis*) turned the urease reagent from orange to pink (5 – 60 min). *Escherichia coli* was used as the negative control (Health Protection Agency, 2010b).

2.8 Oxidase Test

10 µl of the oxidase test solution (Table 2.1) was pipetted onto a single piece of grade 1 filter paper (Whatman). A single colony from a fresh BHI plate was immediately transferred to the area of oxidase solution on the filter paper using a sterile toothpick. A purple colour developed within 10 seconds if the organism was oxidase positive. *Escherichia coli* was used as a negative control and *Pseudomonas aeruginosa* was used as the positive control (Health Protection Agency, 2010a)

2.9 Carbohydrate Metabolism

Oxidative fermentation medium (Table 2.1) was made up using 10 g l^{-1} of one of 10 sugars (glucose, sucrose, fructose, lactose, maltose, mannose, mannitol, galactose, threhalose, xylose, raffinose). 200 µl of medium was then added to each well in a 96-well plate. Pure, single colonies were stab inoculated into the well using a sterile tooth pick. The 96 well plates were then incubated statically for ~18 h. A positive result was given if acid was produced, changing the colour of the pH indicator from blue to yellow.

2.10 Gram Stain

A single bacterial colony was smeared onto a glass microscope slide and gently heat fixed in a Bunsen flame. The slide was then flooded with crystal violet which was rinsed with tap water after 30 sec. The slide was then flooded with 1% (w/v) Lugol's iodine and washed off after 1 min with 95% acetone until the colour stopped running out of the smear. 0.1% safranin was then used to counterstain and was washed off after 1 min. The slide was

then blotted dry and visualized under a microscope (Health Protection Agency, 2007).

2.11 EXO – SAP IT Purification of PCR products

10 µl of ExoSAP- IT® (USB, Staufen, Germany) was added to 50 µl of a PCR reaction and vortexed for 20 seconds. The mixture was then incubated at 37°C for 30 min, followed by 15 min at 80°C to inactivate the Exo-SAP IT.

2.13 Catalase test

10 µl of 5 % (v/v) H_2O_2 was added to a glass microscope slide. A single colony was added to the $H_2O_2$and examined for the production of oxygen bubbles.*S. aureus* served as a calalase positive control and *Streptococcus mitis* was a negative control.

2.14 Nitrate reduction test

An overnight test culture was grown on a BHI agar plate. A loopful of a single colony was added to 1 ml of nitrate media in a 1.5 ml eppendorf, which was then incubated at 37°C for 18 – 24h. After incubation 50 µl of Nitrate reagent A (Table 2.1) was added followed immediately by 50 µl of Nitrate reagent B (Table 2.1). If after 30 sec, a red colour developed the isolate was scored positive for nitrate reduction. A negative result was confirmed by adding powdered zinc to reduce the nitrates to nitrites. A red colour developed after the addition of powdered zinc only in negative tests. *E. coli* was used as the positive control and *S. hominis* (nasal isolate from this study B026) was used as the negative control.

2.15 Phosphatase Assay

Bacteria to be tested were cultured on phenolphthalein phosphate agar plates (Table 2.1) for 18 hrs. Following incubation, 2 ml of 100% (v/v) ammonia (Sigma-Aldrich) was added to the lid of the inverted agar plate and left for 2 min. After this time the colonies on the plate were assessed for phosphatase activity with pink colonies scored as phosphatase positive.

2.16 Lipase activity

Lipase activity was examined by culturing isolates on mannitol salt agar with egg yolk tellurite solution (Table 2.1). Isolates were considered to have lipase activity if a clear zone formed around the colony where the lipase had degraded the egg yolk in the media.

2.17 Biofilm Inhibition Assay

Overnight cultures (method 2.1) of *S. aureus* strain SH1000 were inoculated with a 1% (v/v) inoculum into 96 well plates containing 50% single strength BHI supplemented with 4% (w/v) filter sterilised sucrose (final concentration 2% (w/v)) and 50% spent supernatant from a 18 h old culture of either *S. epidermidis, S. capitis* or *Corynebacterium* sp., up to a total volume of 200 µl. Supernatant from an 18 h culture of strain SH1000 and single strength BHI media were used as controls. Three biological replicates of the culture supernatants were used. The 96 well plates were incubated statically in a humidified environment for 48 h. Following incubation, the media were removed from the 96 well plates by pipetting, taking care not to disturb the

biofilm on the base of the well. The biofilm was then washed 3 times by gently pipetting 200 µl of PBS over the biofilm and removing it by pipetting. The plates were air dried before 50 µl of 0.1% (w/v) crystal violet solution was added to each well and left for 10 minutes to stain the cells in the biofilm. The crystal violet solution was removed and the stained biofilm was washed by pipetting with PBS as previously described. The stained biofilm was then resuspended in 100 µl of 100% (v/v) glacial acetic acid and the optical density was read at 590 nm using a Victor 3 plate reader [Wallac].

2.18 Quorum sensing (Agr) interference

S. aureus strain Liv985 (SH1000 pSB2035 [*agr::luxABCDE]*), which contains the *luxABCDE* genes under the control of the *agr RNAIII* P3 promoter was used to examine Agr signaling interference. Strain Liv985 was incubated in 96 well plates with 50% (v/v) single strength BHI and 50% (v/v) culture supernatant from a test strain. Culture supernatants from *Enterococcus feacalis* (L058 – Liverpool Strain) and *Bacillus cereus* (L001 – Liverpool Strain) were used as negative controls and are not known to produce similar AIPs to *S. aureus* and are unlikely to interfere with Agr signal transduction. *E. faecalis* utilises pheromones which are structurally distinct from the staphylococcal AIP (Nakayama *et al.*, 2001a) and *B. cereus* relies on the competence system, not agr for cell density sensing (Kramer *et al.*, 2007). Single strength BHI medium was also included as a control. Optical density readings (A_{590nm}) and relative luminescence units per second (RLU s^{-1}) were recorded after 7 h when luminescence and thus transcription from *agr* P3 were found to be highest.

2.19 Invasion assay

All nasal isolates were cultured on BHI agar plates prior to the beginning of the invasion experiment. Bacteria were cultured for 18 h on 50 mm diameter BHI agar plates when the lawns of *S. aureus* (SH1000) and *S. epidermidis* strains (resident and invader – Table 6.1) were scraped off the agar plates and suspended in 10 ml of PBS (Table 2.1), (containing approximately 5 x 10^8 cfu ml^{-1} for *S. aureus* and *S. epidermidis*, determined with a colony count) by vortexing thoroughly. The cfu ml^{-1} in each tube was equalised by diluting the cell suspensions in PBS and comparing the OD_{600} of each suspension. The two organisms were then mixed together in a final volume of 10 ml PBS (Table 2.1) with the invader at different frequencies (ratios) to the resident (0.1:1, 0.01:1, 0.001:1). For brevity, these ratios are referred to in this chapter as frequencies and only the first number in the ratio pair is used to define each frequency. The mixtures were vortexed thoroughly before 50 µl (containing approximately 2.5 x 10^6 cells) was plated out on 25 ml BHI agar and incubated at 37° C. Three replicate communities were established at each starting frequency. The communities were transferred to a new agar plate every day for 7 days. Half of the plates underwent a spatial structured regime whereby the transfers were made by replica plating with velvet to maintain spatial structure. While the other half of plates underwent a mixed regime whereby the spatial structure was destroyed at every 24 h transfer by scraping the entire bacterial lawn off the plate and transferring to 10 ml of sterile PBS before thoroughly vortexing and pipetting 50 µl onto a new plate to complete the transfer. Each set was performed in biological triplicate. Viable counts for each isolate were calculated every second day. On the

structured plates this was achieved by scraping the remainder of the bacterial lawn after the replica plating and serial diluting in PBS. Colony counts were done on BHI plates and colonies were differentiated by colony morphology and colour.

2.20 Doubling time determination

An overnight culture of each strain (Table 6.1) was inoculated (1% inoculum) into 200 µl of BHI broth in a 96 well plate. The 96 well plates were incubated at 37°C for 8 h and OD_{600} readings were taken at 20 min intervals. The doubling time (mins) was then calculated using the following formula where T_d is the doubling time; t_1 and t_2 are two consecutive time points throughout the bacterial growth; and d_1 and d_2 are the corresponding OD_{600} readings at t_1 and t_2.

$$T_d = (t_2 - t_1) * \frac{\log(2)}{\log(\frac{d_2}{d_1})}$$

2.21 Bray-Curtis dissimilarity dendrogram

The Bray-Curtis dissimilarity dendrogram was constructed using the "heatmaps.2" package for R. Bray-Curtis dissimilarity was calculated between each pair of 60 bacterial communities based on the presence or absence of species. The Bray-Curtis dissimilarity is the proportion of taxonomically distinct bacterial isolates present in i and/or j that is not present in both i and j. The dendrogram was constructed using complete linkage hierarchical clustering with the "hclust" function in R.

2.22 Principal Components Analysis

Principal components analysis (PCA) was used to separate each different taxonomic unit in multi-dimensional space, based on the amount of variation each dimension could explain. Each dimension explains the maximum proportion of the variation within a sample; the first component explains the greatest variation and each subsequent component explains less of the variation than the one preceding it. For the principal components analysis, the orthogonal transformation of the variables and the plot were produced in the R package Vegan.

2.23 GLM model selection and Statistical test

All statistical analysis was done using R version 2.10.0 (R Development Core Team, 2010). A generalised linear model was used with a logit link function to relate the presence (1) or absence (0) of any given nasal isolate to the presence or absence of *S. aureus*. Significance was attributed to the binomial regression using a Chi-squared test test.

2.24 Calculation of Selection Rate Constant

$$r_{ij} = \frac{ln[N_i(1)/N_i(0)] - ln[N_j(1)/N_j(0)]}{1\,day}$$

Where $\mathbf{N_i\ (0)}$ and $\mathbf{N_j\ (0)}$represent the initial densities of the competing populations I and j and $\mathbf{N_i\ (1)}$ and $\mathbf{N_j\ (1)}$ represent their densities after one day (Travisano & Lenski, 1996).

2.25 Behrens Fisher Multiple comparisons

Behrens Fisher multiple comparison were used to compare several means to the selected control value. The test was controlled for type II errors (alpha = 0.05). A conservative control (i.e. the lowest value) was always chosen for comparison when looking for either biofilm inhibition or Agr interference. The test was therefore constrained to only identify strains with significantly lower means than the control. Higher means could potentially be false positives, as the controls for comparison were selected for their conservatively low value. The analysis was done using the "npmc" package in R (R Development Core Team, 2010).

Media / Buffers / Antibiotic	**Composition**
BHI agar plates	3.7% (w/v) BHI Broth (Lab M), 1.5% (w/v) Agar (Lab M), ddH_2O
BHI broth	3.7% (w/v) BHI Broth (Lab M), ddH_2O
†Chocolate agar	3.7% (w/v) BHI Broth (Lab M), 1.5% (w/v) Agar (Lab M), 5% (v/v) Fresh Horse Blood (E & O Laboratories), ddH_2O
‡Fresh horse blood agar	4.1% (w/v) Columbia Agar Base (Lab M), 5% ‡Fresh Horse Blood (E & O Laboratories), ddH_2O
‡Fresh sheep blood agar	4.1% (w/v) Columbia Agar Base (Lab M), 5% ‡Fresh Sheep Blood (E & O Laboratories), ddH_2O

Mannitol salt agar	10.8% (w/v) Mannitol Salt Agar (Lab M), ddH_2O
‡Mannitol salt agar + egg yolk tellurite	10.8% (w/v) Mannitol Salt Agar (Lab M), 5% ‡egg yolk tellurite solution (Lab M), ddH_2O
Phenolphthalein phosphate agar	4.1% (w/v) Columbia Agar Base (Lab M), 1% (v/v) Phenolphthalein Phosphate (1%)
TAE (50X Stock)	2 M Tris base, 1 M Glacial acetic acid, 0.05 M EDTA
PBS	0.8% (w/v) NaCl, 0.034% (w/v) KH_2PO_4, 0.12% (w/v) K_2HPO_4
0.9% saline	0.9% (w/v) NaCl, ddH_2O
Glycerol for freezer stocks	50 % (v/v) glycerol
Oxidative fermentation medium	2.0 g/l Peptone, 5.0 g/l NaCl, 0.3 g/l K_2HPO_4, 0.015% (v/v) Bromothymol Blue, 10 g/l filter sterilised sugar (dissolved ddH_2O, pH 7.1)
Bromothymol blue	2 g/l (in 50% (v/v) Ethanol)
Phenol red	0.2% (w/v) (in 50% (v/v) Ethanol)
Urease test reagent	0.09% (w/v) KH_2PO_4, 0.095% (w/v) Na_2HPO_4, 2% (w/v) Urea, 0.01% (w/v) Yeast Extract, 5% (v/v) phenol red (pH 6.8)

Nitrate test medium	0.3% (w/v) Beef Extract, 0.5% (w/v) Peptone, 0.1% KNO_3, 1.2% (w/v) Agar (nitrate free)
Nitrate test reagent A	0.5% (w/v) α-Napthylamine, 30% (v/v) acetic acid
Nitrate test reagent B	0.8% (w/v) Sulfanilic acid, 30% (v/v) acetic acid
1% phenolphthalein phosphate	1% (w/v) phenolphthalein diphosphate in 100% (v/v) Ethanol
Oxidase test reagent	1% (w/v) N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloric in ddH_2O
0.5% crystal violet	0.5% (w/v) in ddH_2O
1% Lugol's Iodine	1 % (w/v) Lugol's Iodine in ddH_2O
0.1% safronin	0.1% (w/v) in ddH_2O
5% hydrogen peroxide	5% (v/v) H_2O_2 in ddH_2O
Enzymatic lysis buffer	20 mM Tris.Cl – pH8, 2 mM Sodium EDTA, 1.2% (v/v) Triton X-100

Table 2.1. Components for reagents used throughout this thesis. † Blood component was added to sterilised media and maintained at 48°C for 30 min, or until the erythrocytes were lysed and the media turned from red to brown. ‡ Heat sensitive components were added to sterilised media, which had been tempered to 48°C and plates were cooled quickly in a single layer.

Primer name	Sequence
pA	AGA GTT TGA TCC TGG CTC AG
pH	AAG GAG GTG ATC CAG CCG C

Table 2.2. Sequences of primers used in this thesis. pA and pH are the forward and reverse primers respectively for the 16S rDNA gene. Sequences are from Edwards *et al.*, (1989).

Chapter 3: What is the structure of microbial communities inhabiting the human anterior nares?

3.1 Introduction

Community ecology is the study of the structure and function of biodiversity in natural communities (Morin, 1999). There are three main measures of biodiversity, alpha, beta and gamma, which describe the distribution of biodiversity over different spatial scales (Whittaker, 1972). Alpha diversity measures diversity within an individual community; the simplest measure is species richness, however more complex diversity indices take into account both species richness and the evenness of their abundances. Gamma diversity describes the combined alpha diversity values for communities within a region, thereby representing the regional diversity of a community network (Whittaker, 1972). This also defines a metacommunity (Wilson, 1992). Beta diversity links these spatial scales, describing the dissimilarity in composition between communities (Whittaker, 1972). Gamma diversity is affected by both alpha and beta diversity. A high gamma diversity value could occur if the diversity of the local communities is high, and the difference in diversity between local communities is low (high alpha, low beta) or if the diversity of the local communities is low, but the differences between species present in different local communities is high (low alpha, high beta).

Beta diversity can be used to infer patterns of community structure. Zero beta diversity shows there is no difference between the composition of different local communities, and a non-zero beta diversity shows that there are

fundamental differences between local community structures (Whittaker, 1972). These differences can be either random or non-random. Neutral community models describe how random differences in community structure occur. These models assume that individual species in a metacommunity do not differ in their competitiveness or their suitability for a particular niche (Bell, 2001; He, 2005). Beta diversity is therefore produced by the random allocation of species from the metacommunity pool to different local community patches, so that different community patches have different alpha diversities (Bell, 2001; He, 2005). Beta diversity can also be generated through non-random processes, by which the differences in community structure occur due to heterogeneity in dispersal rate and ecological interactions, such as species habitat interactions and competitive interactions between species (Bell, 2001).

Dispersal is the rate at which a species moves from one local habitat to another and plays a major role in frequency distribution at the local community level. By definition, without dispersal a metacommunity could not exist (Wilson, 1992). Species with lower dispersal rates will typically be found in fewer communities if all other factors are constant (Holt *et al.*, 2003).

Several processes have been identified that give rise to non-random beta diversity in natural metacommunities. Heterogeneity in habitat composition and nutrient distribution often means that certain species from a metacommunity pool are better adapted to specific local community patches (Leibold, 1998). This gives rise to species sorting, where species from the

metacommunity are distributed throughout local communities based on their suitability for a given community patch (Leibold *et al.*, 2004). There are several studies that address this aspect of metacommunity dynamics (Jones & McMahon, 2009; Logue & Lindstrom, 2010; Pillon *et al.*, 2010). For species sorting to occur in natural communities, dispersal has to be high enough to ensure that the most suitable species can fill the most suitable niches. Mass effects models consider the scenario where dispersal is so high that local dynamics can be overridden, and species can occupy niches for which they are not suitably adapted, such as a black hole sink habitats which would not otherwise support viable populations (Lee & Gelembiuk, 2008).

In addition to heterogeneity in habitat composition, interactions between species can strongly influence community structure and beta diversity. Species interactions may be direct, such as competition or mutualism (Morin, 1999), or indirect, such as apparent competition brought about through an intermediary (Frid & Marliave, 2010). The effect of species interactions has been modelled and named the patch dynamic paradigm (Leibold *et al.*, 2004). Early models considered resource competition as the only species interaction (Levins & Culver, 1971). However, more complex models were developed to additionally include predator-prey interactions, which can be viewed as interference competition in microorganisms (Caswell, 1978; Crowley, 1981; McCauley *et al.*, 1993). Patch dynamic models tend to assume that there is a competition-colonisation trade-off. That is to say, that most species are either good colonisers or strong competitors, but cannot do both optimally (Tilman, 1994; Yu & Wilson, 2001; Yu *et al.*, 2001).

Inferences about species interactions can be made from metacommunity data, although the specific mechanism of the interactions can be much harder to elucidate. Another approach used to infer interactions is ordination. Ordination is a method of clustering variables based on their similarity (Beals, 1965; Bray & Curtis, 1957; Gittins, 1965). In the study of ecology, species coexisting in the natural environment, will cluster together in an ordination analysis, just as species that don't coexist, will not cluster together (Queirolo *et al.*, 2011; Rudner, 2011; Schotthoefer *et al.*, 2011). Ordination has been used recently to interpret the microbial communities of the human anterior nares (Frank *et al.*, 2010; Wos-Oxley *et al.*, 2010).

3.2 Aims

The aim of this chapter was to representatively survey the metacommunity of the human anterior nares by sampling 60 volunteers (representing 60 different local communities). Subsequent analysis of the metacommunity was carried out in order to infer interactions between species, based on their distribution patterns throughout the local communities.

3.3 Materials and Methods

3.3.1 Bacterial growth conditions

The *S. aureus* strains and all of the nasal isolates from this study were cultured in 10 ml BHI broth shaken at 200 rpm and on agar solidified BHI medium (Lab M) at 37°C. Chemicals were obtained from Sigma-Aldrich Co., United Kingdom, unless otherwise stated.

3.3.2 Isolation of cultivable nasal microbiota from healthy volunteers

Ethical approval for the isolation protocol was granted by the University of Liverpool Ethics Committee, licence number RETH000089, 8th November 2007. The left and right anterior nares of 60 volunteers were sampled using cotton swabs (Technical Service Consultants Ltd) under a standardised sampling regimen. The left and right swabs were placed together in 2 ml of 0.9% (w/v) saline, vortexed for 60 seconds and 100 µl was plated onto four different agars: chocolate agar (BHI supplemented with 5% (v/v) horse blood), sheep blood agar (Columbia agar base supplemented with 5% (v/v) sheep Blood), mannitol salt agar supplemented with egg yolk tellurite and BHI agar (Table 2.1). Plates were incubated at 37°C for 48 h before enumeration and identification. Colonies were initially differentiated on chocolate agar plates after 48 h, since this medium generated the greatest phenotypic diversity. Between 5 and 10 colonies of each visually distinct colony morphology were picked and subjected to an initial round of phenotypic screening to elucidate whether they were indeed identical. The phenotypic screen included 11 carbohydrate metabolism tests using Hugh

and Leifson's medium (Hugh & Leifson, 1953) (glucose, sucrose, fructose, lactose, maltose, mannose, mannitol, galactose, threhalose, xylose, raffinose) (method 2.9), urease activity (method 2.7), catalase activity (method 2.13), haemolysis on sheep blood agar (Table 2.1) and lipase activity (method 2.16). Each phenotypically distinct isolate was then stored at -80C prior to species identification.

3.3.3 Identification of bacterial isolates

Presumptive staphylococcal isolates were identified using API staph (BioMérieux, Marcy-l'Etoile, France). For all other isolates, and where API STAPH failed to provide an identity, 16S ribosomal DNA sequencing was used for identification. Genomic DNA was extracted from a 1.5 ml aliquot of a 10 ml overnight culture. The cells in the 1.5 ml aliquot were harvested by centrifugation and resuspended in lysis buffer supplemented with 20 mg ml^{-1} lysozyme and 20 mg ml^{-1} lysostaphin, specficially for Gram-positive organisms before genomic DNA extraction using a DNeasy Blood and Tissue Kit (Qiagen). The 16S gene was amplified using primers pA and pH (Edwards *et al.*, 1989) with BioMix Red polymerase (Bioline) supplemented with 2.5 U ACCUZYME™DNA Polymerase (Bioline) according to manufacturer's instructions using the following PCR cycling conditions: denaturation at 95°C for 5 min, followed by 30 cycles [95°C for 1 min, 55°C for 1 min, 72°C for 1.5 min] and a final extension at 72°C for 7 min. The PCR products were checked for purity by electrophoretic separation on a 1% (w/v) TAE agarose gel (agarose from Bioline) run in TAE buffer for 45 minutes at 80 V. A 30 µl aliquot of the PCR reaction was incubated with ExoSAP-IT®

(USB,Staufen, Germany) to dephosphorylate primers and dNTPs according to the manufacturer's instructions. The treated PCR product was then sequenced using the forward pA primer by GATC (Konstanz, Germany) using the Sanger method on an ABI 3730xl sequencing machine. DNA sequences were analysed with the Sequence Match program on the RDP website (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) (Michigan State University) to identify the closest matching sequences. Identities were assigned based on the matching sequence with the lowest e-value, i.e. the lowest chance of a match occurring by chance, accounting for the quality and length of the sequence, as well as the size of the database. Each identity was assigned only if there were no other identities in the database with the same e-value. Any ties in the e-value were re-sequenced.

3.3.4 Statistical analysis

All statistical analysis was done using R version 2.10.0.

The Bray-Curtis dissimilarity dendrogram was constructed using the "heatmaps.2" package for R. Bray-Curtis dissimilarity was calculated between each pair of 60 bacterial communities based on the presence or absence of species. The Bray-Curtis dissimilarity is the proportion of taxonomically distinct bacterial isolates present in i and/or j that is not present in both i and j. The dendrogram was constructed using complete linkage hierarchical clustering with the "hclust" function in R.

Principal components analysis was used to separate each different taxonomic unit in multi-dimensional space, based on the amount of variation each dimension could explain. Each dimension explains the maximum

proportion of the variation within a sample; the first component explains the greatest variation and each subsequent component explains less of the variation than the one preceding it. For the principal components analysis, the orthogonal transformation of the variables and the plot were produced in the R package Vegan.

Logistic regression is a powerful way to analyse binary data with many different predictor variables. As such it was used to ascribe significance to the presence / absence data and to show whether any given pair of taxonomic units were positively or negatively associated. A generalised linear model with a logit link function was used to find a fit for the species' distributions. The significance of the fits were then analysed using a Chi Squared test.

3.4 Results

To address whether the nasal microbiota play a role in determining *S. aureus* carriage, bacterial communities were sampled from the noses of 60 healthy human volunteers. Swab samples from each nostril of a volunteer were pooled and plated on rich media (chocolate agar, BHI, sheep blood agar) to cultivate a broad range of taxa and allow enumeration of bacterial density. In addition, sheep blood agar and mannitol salt agar supplemented with egg yolk tellurite were used as indicator media to allow discrimination of staphylococci. Multiple isolates (between 5 and 10) of each phenotypically distinct colony type were picked and streak-plated to purity on Chocolate Agar. Bacteria were phenotypically differentiated using a series of carbohydrate metabolism indicator tests, assaying urease catalase and lipase activity and haemolysis on Sheep blood agar (method 3.3.2). Presumptive staphylococci from these tests and as determined by Gram stain and microscopic morphology, were identified to species-level using API Staph, while other taxa were identified by 16s rRNA gene sequencing.

39 taxonomic groups were isolated from the 60 independent nasal communities (Fig. 3.1 & Appendix 1 table 8.1). Mean species richness per nose was 3.15 (range: 1 to 5 species). There was no significant correlation between total bacterial density and species richness (Fig. 3.2.A; Spearman's $r = 0.16$, $N = 60$, $p = 0.22$), and mean species richness of *S. aureus* containing communities was not significantly different from communities lacking *S. aureus*, ($T = 0.65$, $df = 56$, $p = 0.517$) suggesting that sampling was relatively unbiased and representative.

The regional frequencies of the taxa were highly skewed (Fig. 3.1). 52.9% of the observed regional frequencies were accounted for by only 4 taxa, and the 20 least abundant taxa were only present in one sample each and accounted for a total of 10.6% of the sampled taxa. This skew of the regional frequency (Fig. 3.1) could be explained by the core satellite hypothesis (Hanski, 1982), which states that most taxa in a metacommunity are either highly abundant or very rare, giving rise to a bimodal taxon frequency distribution. From this distribution, taxa present at high frequency can be considered core taxa, and those at low frequency may be considered satellite taxa.

The metacommunity structure was examined to investigate if a subset of core and satellite taxa could be identified. There was a significant positive regional frequency-local abundance relationship (Fig. 3.2.B: log transformed linear regression, $r^2 = 0.535$, $p < 0.001$), which is an important assumption of the core satellite hypothesis. The regional frequency distribution of the taxa is also bimodal, with 28 taxa occupying less than 25% (23.3%) of the communities, and 11 taxa occupying more than 75% (76.7%) of the nasal communities (Fig. 3.2.C). A discontinuity in the frequency abundance distribution (Fig. 3.2D) between 10 and 30 occurrences, defines the core taxa as those present in greater than 10 communities and satellite taxa present in less than 10 (Magurran *et. al.* 2003). Using this method, *S. epidermidis* and *S. aureus* are the only core taxa, being present in 51 and 29 of the samples respectively; all other taxa were considered rare and therefore satellite. Satellites were not removed from subsequent analysis as organisms known to interact with *S. aureus* (namely *Corynebacterium* sp) (Uehara *et. al.* 2000) were present in this group.

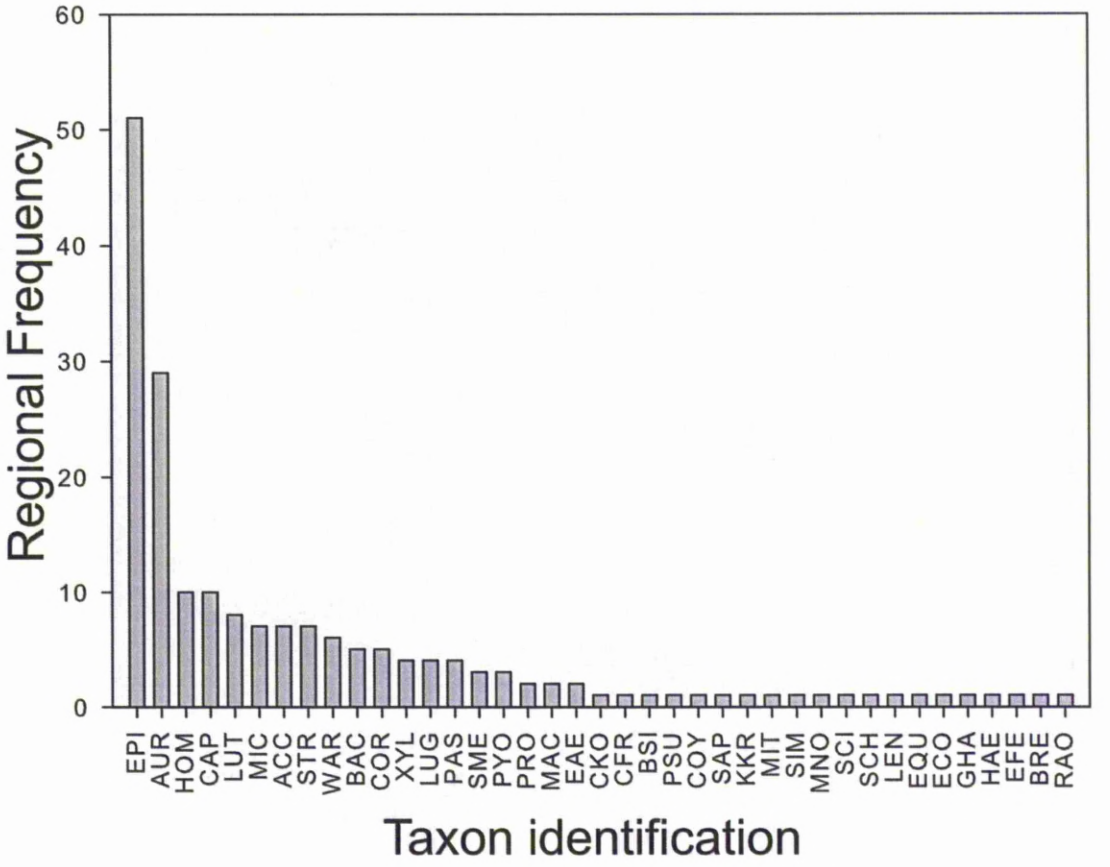


Figure 3.1. Regional frequency distribution of all 39 taxa ranked from largest to smallest. The taxon identification is a 3 letter code denoting each taxon as follows: EPI, *S. epidermidis;* AUR, *S. aureus;* HOM, *S. hominis;* CAP, *S. capitis;* LUT, *Micrococcus luteus;* MIC, *Micrococcus* sp.; ACC, *Corynebacterium accolens;* STR, *Streptococcus* sp.; WAR, *S. warneri;* BAC, *Bacillus* sp.; XYL, *S. xylosus*; LUG, *S. lugdunensis;* PAS, *S. pasteuri;* SME, *Corynebacterium smegmentosum;* PYO, *Streptococcus pyogenes;* PRO, *Corynebacterium propinquum*; MAC, *Corynebacterium macginleyi;* EAE, *Enterobacter aerogenes;* CKO, *Citrobacter koseri;* CFR, *Citrobacter freundii;* BSI, *Bacillus simplex;* PSU, *Corynebacterium pseudodiptheriticum;* COY, *Corynebacterium coyleae;* SAP, *S. saprophyticus;* KKR, *Kocuria kristinae;* MIT, *Streptococcus mitis;* SIM, *S. simulans;* MNO, Moraxella nonliquefaciens; SCI, *S. sciuri;* SCH, *S. schleiferi;* LEN, *S. lentus;* EQU, *S. equorum;* ECO, *Escherichia coli;* GHA, *Gemella haemolysans;* HAE; *S. haemolyticus;* BRE, *Brevidobacterium* sp., RAO, *Raoultella* sp.

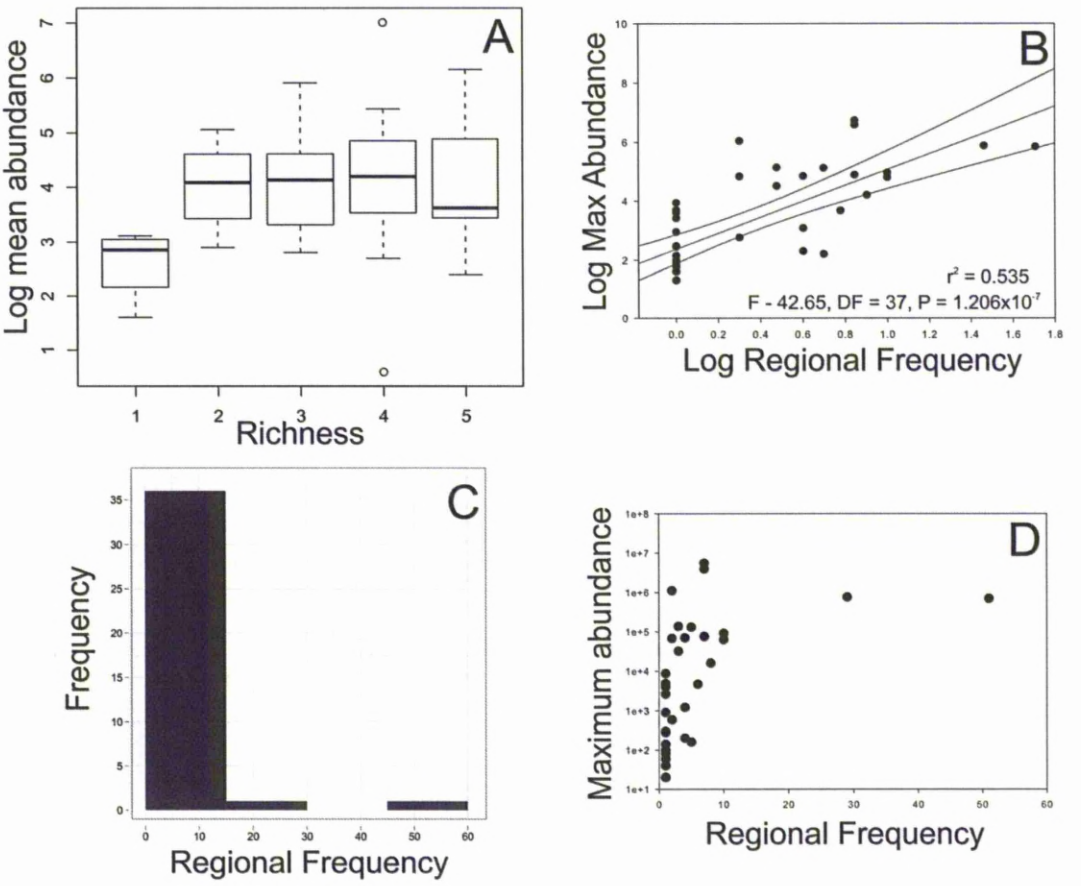


Figure 3.2. Metacommunity Analysis. (A) Log mean abundance against species richness for each community showing a positive, but insignificant, correlation (Spearman's r = 0.16, N = 60, p = 0.22) indicating saturation of abundance at higher taxa richness. (B) Relationship between local abundance and regional frequency. Both values are log transformed and maximum local abundance is used instead of the mean due to the non – normal distribution of abundance values. (C) Frequency histogram showing a bimodal taxa distribution. Occurrences of taxa in this sample tend to be explained by many satellite taxa occurring in few samples (first peak) or few core species occurring in many samples (second peak). (D) A second plot of the maximum local abundance (x-axis, log scale) against regional frequency (non-log transformed) to determine the cut off between core and satellite species. The natural break in the distribution between 10 and 30 defines the core species and >10 and satellite species ≤10.

Cluster analysis was performed to determine community dissimilarity, i.e. beta diversity (Whittaker, 1972), and to elucidate whether community composition had an effect on the presence or absence of *S. aureus*. Figure 3 shows a Bray – Curtis dissimilarity dendrogram with 80% and 50% dissimilarity marked with a dashed line. Given the large number of groups at 80% dissimilarity that contained *S. aureus* (AUR), it is difficult to infer interactions between taxa using this method. However, it is noteworthy that three of the groups at 50% dissimilarity (Fig. 3.3 - C, G & H) are not *S. aureus* (AUR) containing communities. Moreover, every community in these three groups (Fig.. 3.3 - C, G & H) contained a strain of *S. epidermidis* (EPI). Furthermore, every community in group C at 50% dissimilarity also contained *Micrococcus* sp. (MIC) and every community in group H contained *C. accolens* (ACC). *S. epidermidis* was the only common taxon among the communities of group G (Fig. 3.3).

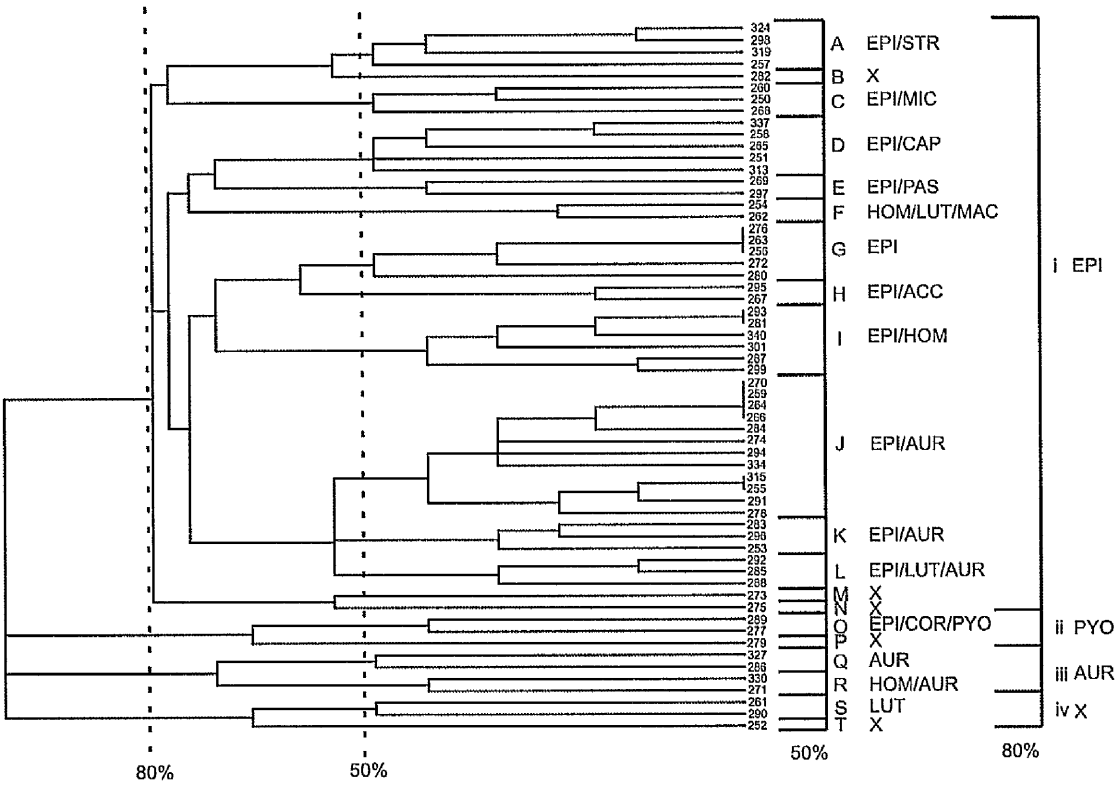


Figure 3.3. Bray – Curtis cluster analysis of nasal communities. The dashed lines represent 80% dissimilarity and 50% dissimilarity cutoffs. These cut offs correspond to the cluster descriptions at the right of the dendrogram. The 3 letter taxa codes (from Fig. 1. legend) to the left of the dendrogram signify if a taxon was present in every community within a cluster and hence partially responsible for the formation of the cluster. X signifies that there were no common taxa in every community in the cluster. The taxon identification is a 3 letter code denoting each taxon as follows: EPI, *S. epidermidis;* AUR, *S. aureus;* HOM, *S. hominis;* CAP, *S. capitis;* LUT, *Micrococcus luteus;* MIC, *Micrococcus* sp.; ACC, *Corynebacterium accolens;* STR, *Streptococcus* sp.; WAR, *S. warneri;* BAC, *Bacillus* sp.; XYL, *S. xylosus*; LUG, *S. lugdunensis;* PAS, *S. pasteuri;* SME, *Corynebacterium smegmentosum;* PYO, *Streptococcus pyogenes;* PRO, *Corynebacterium propinquum*; MAC, *Corynebacterium macginleyi;* EAE, *Enterobacter aerogenes;* CKO, *Citrobacter koseri;* CFR, *Citrobacter freundii;* BSI, *Bacillus simplex;* PSU, *Corynebacterium pseudodiptheriticum;* COY, *Corynebacterium coyleae;* SAP, *S. saprophyticus;* KKR, *Kocuria kristinae;* MIT, *Streptococcus mitis;* SIM, *S. simulans;* MNO, Moraxella nonliquefaciens; SCI, *S. sciuri;* SCH, *S. schleiferi;* LEN, *S. lentus;* EQU, *S. equorum;* ECO, *Escherichia coli;* GHA, *Gemella haemolysans;* HAE; *S. haemolyticus;* BRE, *Brevidobacterium* sp., RAO, *Raoultella* sp.

Ordination (Principal components analysis; PCA) was used to further explore interactions between taxa. The variances of the taxa distributions were used to reduce the observations in the data set to the smallest number of uncorrelated variables (components) that explain as much of the variation as possible. Principal Component 1 (PC1) in figure 3.4.A. explains 7.7% of the variation and Principal component 2 (PC2) explains 6.1%. The Euclidean distances from *S. aureus* (AUR) to all of the other taxa are plotted for PC1 (Fig. 3.5.A), PC2 (Fig. 3.5.B) and the combined distance of both PC1 and PC2 (Fig. 3.5.C). On PC1 (Fig. 3.5.A), *Corynebacterium propinquum* (PRO), *S. lentus* (LEN) and *S. haemolyticus* (HAE) were most distant from *S. aureus*. On PC2 (Fig. 3.5.B), *S. epidermidis* (EPI) is the most distant taxon from *S. aureus*. These four species are also the most distant when combining the two Principal Component Euclidean distances, with the addition of *Streptococcus* spp. (STR) (Fig. 3.5.C).

PCA was further employed to investigate genus level interactions. The genera were grouped by colour in Figure 3.4.B and a 95% confidence interval ellipse was drawn around the members of each genus. All the genera groupings overlapped, indicating there were no genus level negative associations. However, some taxa fell outside of the 95% confidence interval ellipses of their genera, suggesting a negative association between these taxa and members of their genera (e.g. *S. aureus* (AUR) and *S. epidermidis* (EPI) Fig. 3.4.B). Interestingly, some taxa, including *S. aureus*, and the taxa with the furthest Euclidean distances from *S. aureus* (Fig. 3.5, A, B, C), fell outside of the 95% confidence ellipse of their own genus' cluster (Fig. 3.4). This suggests that genus-level identifications may not be a good predictor of

the ecological role of a given species within the nasal microbial metacommunity.

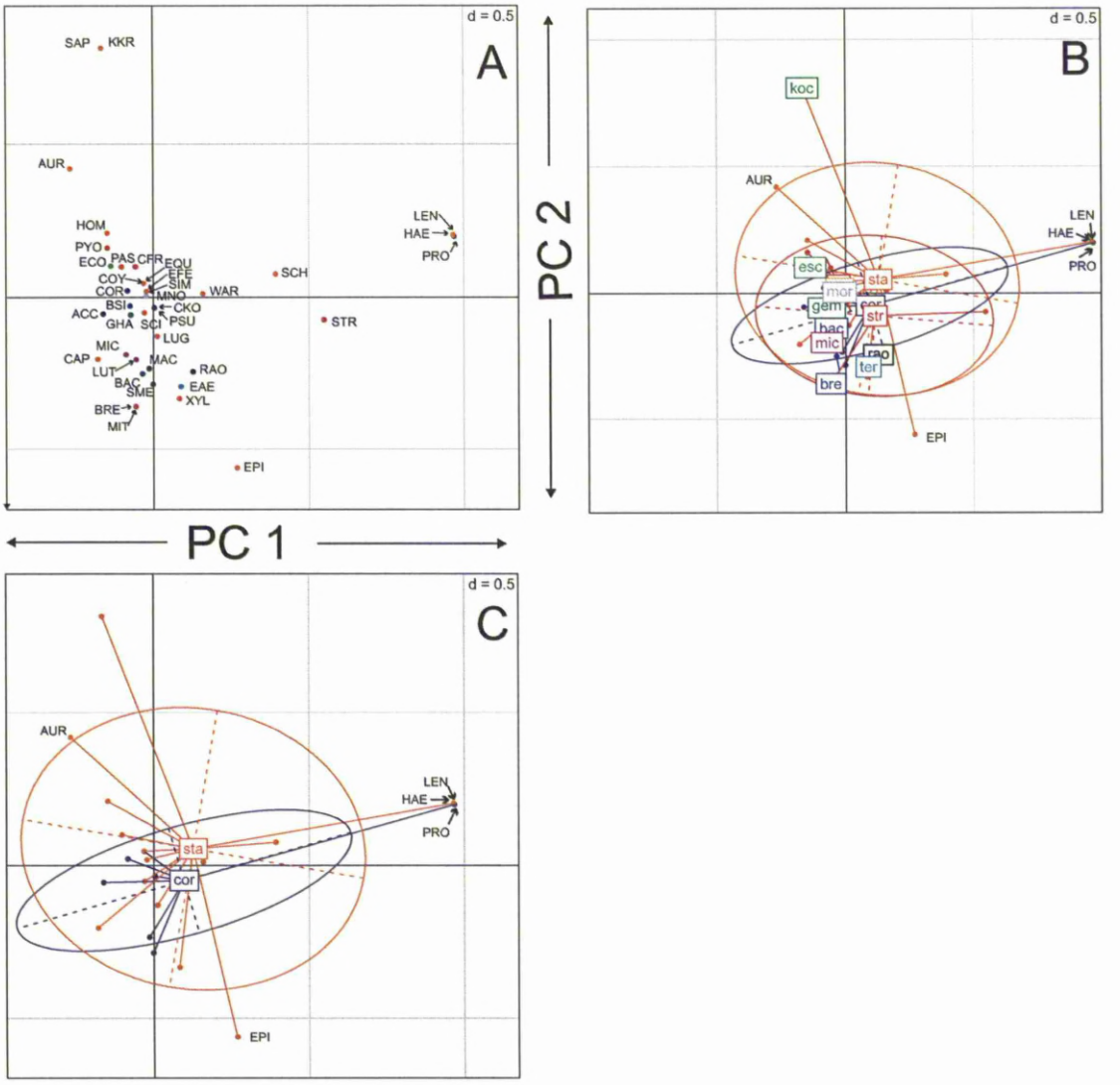


Figure 3.4. Ordination plots of the taxa spaced apart basted on their distribution throughout the nasal communities. The further apart taxa appear in the plot, the less likely they are to be found in the same community. Panels A, B & C show Principal Components Analysis, Principal Component 1 (PC1) plotted against Principal Component 2 (PC2), the black lines crossing in the centre of each plot represent the origin (Euclidean distance of 0,0) and the Euclidean distance between each of the grey gridlines is 0.5. Panel A shows the simple ordination plot for all the taxa. Panel B shows the same plot with 95% confidence interval ellipses drawn around taxa belonging to the same genus. Panel C shows only the genera *Staphylococcus* (sta) and *Corynebacterium* (cor) with the 95% confidence interval ellipses.

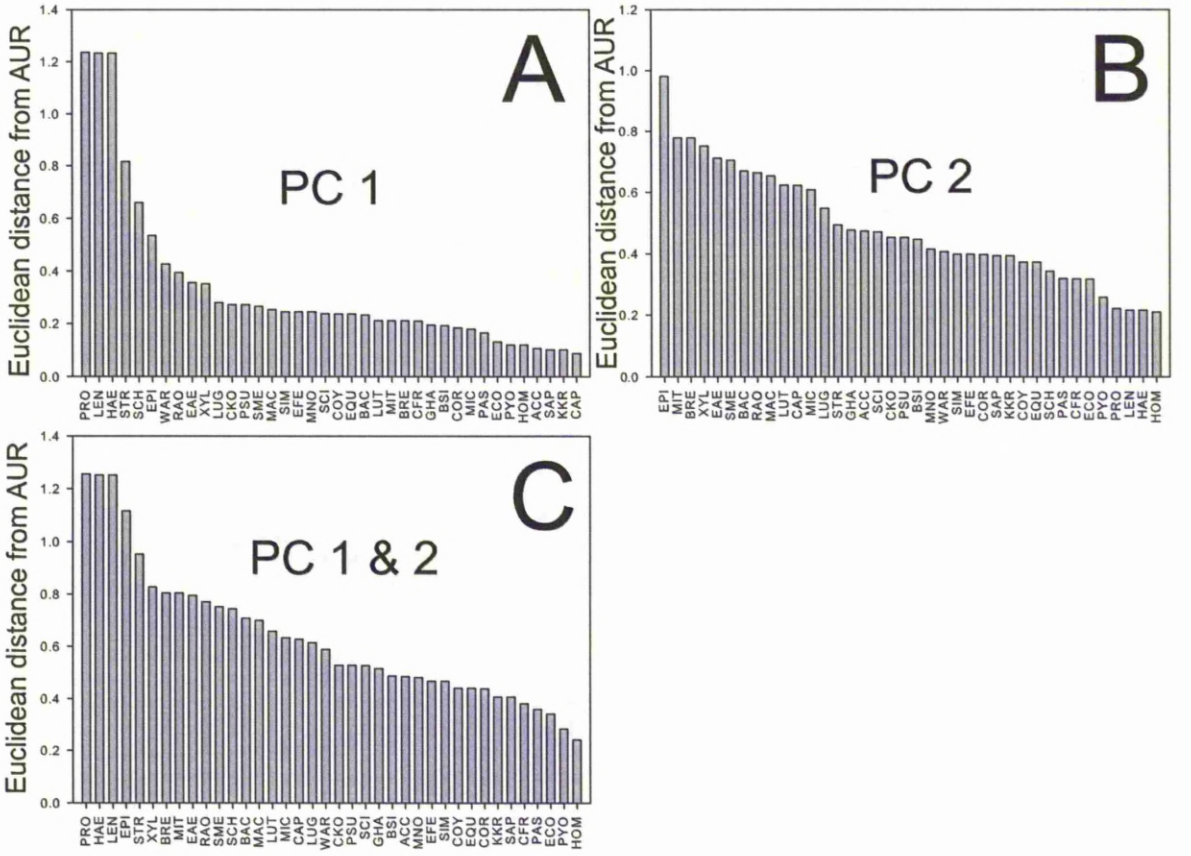


Figure 3.5. Ranked Euclidean distances between *S. aureus* (AUR) and all other taxa from Principal components analysis (Fig. 3.4). Euclidean distances are shown for principal component 1 (panel A), principal component 2 (panel B) and the two components combined using the formula $(dPC1)^2 + (dPC2)^2 = (d.total)^2$, where d is the distance along each axis (panel C).

A Generalised Linear Model (GLM) with a logit link function was used to detect statistically significant species associations. The significance of the fit of the GLM was measured using a Chi-Square test (method 2.17). Two models were fitted and in the first unweighted model (Table 3.2.A) taxa were weighted equally based on presence or absence from an individual community. In the second model taxa were weighted based on their local abundance (Table 3.2.B). Weighting the data gave more importance to species with a higher local abundance. Significance in either model indicates that the distribution of the taxon is significantly different from the distribution of *S. aureus.*

Four taxa were significantly negatively-associated with *S. aureus* in the unweighted model (Table 3.2.A). *Corynebacterium propinquum* PRO, *Corynebacterium macginleyi,* (MAC) and *Enterobacter aerogenes* (EAE) were all significant ($p<0.05$) but the most highly significant negative association was with *S. capitis* (CAP, $p <0.01$). Eight taxa were significantly negatively-associated in the weighted model (Table 3.2.B). *S. epidermidis* (EPI), *Micrococcus* sp. (MIC), *Bacillus* sp. (BAC), *Corynebacterium accolens* (ACC) and *Gemella haemolysans* (GHA) associations were all significant ($p<0.05$) but (CAP) and (SCH) were the most significant negative associations. *S. capitis* (CAP) and *Enterobacter aerogenes* (EAE) are the only taxa to be significantly negatively-associated with *S. aureus* in both models. However as a genus, several *Corynebacterium* sp. were also significantly negatively-associated with *S. aureus* in each model. There were also several staphylococci with significant negative associations with *S. aureus* which could be expected from the PCA analysis (Fig.. 3.4) in which *S.*

aureus falls outside the 95% confidence ellipse of the *Staphylococcus* genus. The aforementioned *S. capitis* (CAP) was significantly negatively-associated in both GLMs, whereas *S. epidermidis* (EPI) and *S. schleiferi* (SCH) were only significantly associated in the weighted GLM (Table 3.2.B).

	Df	Deviance	AIC	LRT	Pr(Chi)
HOM	1	60.45	78.45	3.00	0.0830
CAP	1	66.39	84.39	8.94	**0.0028
CKO	1	60.09	78.09	2.64	0.1043
PRO	1	62.57	80.57	5.12	*0.0237
PSU	1	60.09	78.09	2.64	0.1043
MAC	1	62.57	80.57	5.12	*0.0237
MNO	1	60.09	78.09	2.64	0.1043
EAE	1	62.57	80.57	5.12	*0.0237
RAO	1	60.09	78.09	2.64	0.1043

Table 3.2.A

	Df	Deviance	AIC	LRT	Pr(Chi)
EPI	1	46.94	72.94	4.38	*0.0363
MIC	1	48.19	74.19	5.64	*0.0175
CAP	1	50.57	76.57	8.01	**0.0046
WAR	1	44.89	70.89	2.34	0.1263
BAC	1	48.19	74.19	5.64	*0.0176
PRO	1	46.16	72.16	3.61	0.0574
PSU	1	45.45	71.45	2.90	0.0886
ACC	1	46.89	72.89	4.33	*0.0374
STR	1	45.11	71.11	2.55	0.1100
SCH	1	1585.92	1611.92	1543.37	***<2.2e-16
GHA	1	49.17	75.17	6.61	*0.0101
EAE	1	47.34	73.34	4.79	*0.0286
RAO	1	45.30	71.30	2.75	0.0973

Table 3.2.B.

Table 3.2. GLM results of the unweighted, and weighted binary taxa distributions. The distributions among all of the samples of each taxa were compared to the distribution of *S. aureus* using simple presence or absence data (A) or by weighting the presence / absence data with the local abundance (B). The likelihood (Method 2.23) ratio test (LRT) and p-values (Pr(Chi)) show the significance of the distributions along with the Akaike Information Criterion which quantitatively demonstrates the suitability of this model, by ranking it against alternative models which had a higher variance and were less accurate and were hence not used (method 2.23).

3.5 Discussion

Examination of the cultivable members of the nasal microbiota has revealed several taxa that can potentially exclude *S. aureus* from the niche. These are: *S. capitis, C. propinquum, C. macginleyi, Enterobacter aerogenes, S. epidermidis, Micrococcus* sp., *Bacillus* sp., *C. accolens, S. schleiferi* and *Gemella haemolysins.* The two most predominant genera resulting in a significant negative association are *Staphylococcus* and *Corynebacterium.*

Some of the associations between species revealed in this study have been previously documented. *S. epidermidis* is known to have a negative interaction with *S. aureus* and this has so far been proposed to occur via two potential mechanisms, namely ESP mediated biofilm disruption (Iwase *et al.*, 2010), and *agr* interference (Lina *et al.*, 2003) (investigated further in chapter 5). Undefined *Corynebacterium* species have been shown previously to be negatively associated with *S. aureus.* (Uehara *et. al.* 2000). However, the present study identifies that three species of *Corynebacterium* in particular might account for this negative interaction, namely *C. propinquum, C. macguinleyei* and *C. accolens.* This latter species was shown to be negatively associated with *S. aureus* by Wos-Oxley *et. al.* (2010) using a SSCP analysis. In the present study *C. accolens* was shown to be present in 7 out of 60 noses, which is a higher proportion than for both *C.propinquum* (2 out of 60) and *C. macguinleyei* (1 out of 60) (Fig. 1). Despite *C. propinquum* and *C. macguinleyei* being present in relatively few samples they were present in relatively high abundance in their respective communities (Table 3.1).

S. capitis (present in 10 out of 60 noses) was significantly negatively associated with *S. aureus* but has never before been implicated in affecting *S. aureus* carriage (Table 3.2). While it may have an antagonistic effect on *S. aureus* as described with *S. epidermidis* (Iwase *et al.*, 2010) and a *Corynebacterium* sp (Uehara *et al.*, 2000), it may also be the case that *S. capitis*, is simply better adapted to utilise resources in the niche to the detriment of *S. aureus*. Possible mechanisms for the negative association will be investigated in the following chapters. Interference competition (chapter 4), agr signal disruption (chapter 5) and prevention of biofilm formation (chapter 5) are all discussed as possible causes of the negative association.

Two other previously undescribed negative associations with *S. aureus* but identified here are *Gemella haemolysans* and *Enterobacter aerogenes*. There are no accounts of these species being negatively associated in the scientific literature to date. Their occurrence in this study was very low, with *Gemella haemolysans* only being present in 1 out of 60 samples and *Enterobacter aerogenes* being present in 2 out of 60. However, they make up a substantial proportion of each community in which they reside which explains why both organisms were found to be significantly negatively associated with *S. aureus* in the GLM (Table 3.3) weighted with the abundance data (Table 3.1). This is an interesting result however the low frequency of both species in our sample makes it difficult to draw definitive conclusions regarding these interactions.

The discovery of interactions between different taxa in this chapter and in other studies has potential implications in the design of decolonisation strategies and prevention of *S. aureus* infection. There is now a growing body of evidence to suggest that interactions between taxa can determine carriage of *S. aureus* in the niche (Frank *et al.*, 2010; Heczko *et al.*, 1981; Iwase *et al.*, 2010; Selva *et al.*, 2009; Uehara *et al.*, 2000; Wos-Oxley *et al.*, 2010). At the very least, this research suggests that considerations of the nasal microbial community should be made when administering antimicrobial therapies, as the effect of drugs on this community could change the composition and therefore the potential community interactions. However, it may also be possible to harness the microbial community as a therapeutic in its own right. If it is possible to understand which community compositions exclude *S. aureus,* these communities could be propagated in the niche as a method of decolonisation. This idea has been extensively studied in microbial communities of the human gut (Baines *et al.*, 2009; Baines *et al.*, 2011; Collins & Gibson, 1999). By providing a source of beneficial bacteria (probiotics) or substrates that promote the proliferation of the desired microbial taxa (prebiotics), it may be possible to alter the microbial community composition (Collins & Gibson, 1999). Intriguingly, the pneumococcal vaccine has been correlated with an increase in *S. aureus* otitis media infections, suggesting that this was potentially caused by a shift in the microbial community (Bogaert *et al.*, 2004). Therefore an understanding of the microbial communites is required as such shifts in community composition can have unforeseen complications via increased

disease. Formulations of new drugs and vaccines would need to be tested to prevent undesirable community shifts from occurring.

While it is important to find interactions this is clearly very challenging, exemplified by the fact that Uehara *et. al.* (2000) demonstrated the antagonism by *Corynebacterium* spp. over a decade ago, and no mechanism has yet been identified. This is in part due to the fact that known mechanisms for antagonism are so diverse and possibly function somewhat differently in the nasal environment. Even the studies by Uehara, *et. al.* (2000) and Iwase *et. al.* (2010) which demonstrate displacement of *S. aureus* from the human nares, may not accurately represent the natural environment due to the extremely high innoculum used (1×10^9 cells per nose), when the mean bacterial density sampled from each nose in this survey was only 8×10^4.

An unexpected result from this survey was the absence of *Propionibacterium* species. This is likely attributable to the relatively short time that the agar plates were incubated after being spread with the nasal isolates. It is not thought to be due to the facultative anaerobic or strict anaerobic nature of some *Propionibacterium* species as the sampling method used did not sample any anoxic regions of the anterior nares (i.e. hair follicle root) and the samples were incubated in 5% CO_2, and using anaerobic jars (Oxoid). Furthermore, a study by Rasmussen *et al.* (2000) focussed exclusively on the aerobic members of the nasal microbial community and *Propionibacteria* species were isolated. The culture independent studies of the nasal vestibule (Frank *et al.*, 2010; Wos-Oxley *et al.*, 2010) both show that *Propionibacteria*

spp are underrepresented in culture dependent studies which is certainly true in this study, however, the work by Wos-Oxley *et. al.* also suggests that current culture independent methods may over represent *Propionibacterium* species in their samples. This insight comes from their comparisons between the ribosomal DNA and ribosomal RNA taken from a selection of their samples. The DNA of *Propionibacteria* species was always more abundant in the DNA sample than the RNA, indicating that though *Propionibacterium* spp can be detected, they may not be active or even viable.

The bacterial species represent an obvious and convenient category for grouping strains of bacteria together to test whether they exclude *S. aureus,* however, there may be factors causing exclusion of *S. aureus* that are not associated with a specific taxon. Factors such as bacteriocin production, resource sequestration or growth rate can differ within a taxon and yet all could be important factors in excluding *S. aureus*. Moreover, many antagonistic defences are located on mobile genetic elements that can be horizontally transferred, even between different species (de la Cruz & Davies, 2000). Larger studies may begin to capture aspects of how different phenotypes and properties of interference competition are distributed within and between taxa.

This chapter highlights some novel and some previously described associations between members of the nasal microbial community and *S. aureus*. A logical next step was to take advantage of the culture dependent methods used, and to try to elucidate mechanisms for apparent associations.

By culturing the organisms, phenotypes and interactions could be investigated *in vitro* to determine if these might affect *S. aureus* carriage.

Chapter 4: Does interspecific toxin-mediated interference competition contribute to the distribution of *S. aureus* across communities?

4.1 Introduction

After this study had commenced meta-community analyses of the human anterior nares were reported using 16S rDNA pyrosequencing technologies to elucidate the composition and structure of microbial consortia (Frank *et al.*, 2010; Wos-Oxley *et al.*, 2010). These studies revealed that culture dependent approaches consistently underestimate diversity and richness of microbial communities due to the problems associated with sampling and culturing viable organisms. However, because of a lack of direct experimental tests, current culture independent methods cannot elucidate the mechanisms that underpin the observed community structure. Culture independent studies fail therefore to address the importance interactions between species within a community in driving community structure.

Interspecific competition between organisms can influence the dynamics and composition of communities. Competitive interactions are dichotomously classified into either exploitative competition (MacLean & Gudelj, 2006; Vance, 1984), which involves common resource depletion, or interference competition (Amarasekare, 2002), which in microbes typically involves chemically antagonistic interactions via secreted molecules, also termed interference competition (Morin, 1999). Theoretical and empirical studies suggest that interference competition alleviates resource competition by

removing competitors from a given niche (Garbeva *et al.*, 2011). Furthermore, allelopathy can act to promote invasion by allelopathic species into niches occupied by susceptible species (Chao & Levin, 1981).

Interference competition is widespread and well documented among microbes and can take three main forms, toxic secondary metabolites (Dong *et al.*, 2011; Guroy *et al.*, 2011; Leão *et al.*, 2009), antimicrobial peptides (Gardner *et al.*, 2004; Jack *et al.*, 1995) and antibiotics (Rasool & Wimpenny, 1982; Turpin *et al.*, 1992; Wiener, 2000). Although antibiotics were initially described as any compound which has the potential to negatively impact on the fitness, fecundity or survival of another organism (Davies, 1990), there are important differences between the three forms. The strictest definition is for the antimicrobial peptides produced by bacteria called bacteriocins (Jack *et al.*, 1995), which are ribosomally synthesised peptides (Nissen-Meyer & Nes, 1997). Antibiotics cover a broader range of diverse chemicals and can be defined as a compound that is inhibitory at low concentrations (Jack *et al.*, 1995). This definition also includes peptides which can be classed as antibiotics if they are not ribosomally synthesised, but synthesised by multi-enzyme complexes (Kleinkauf & Vondohren, 1990). The definition of toxic chemicals is the broadest of the three and covers any other toxic secondary metabolite produced by bacteria. Indeed most microorganisms have the potential to produce compounds which are inhibitory to themselves and potential competitors if the concentration is high enough (Tagg *et al.*, 1976).

Bacteriocins are ribosomally synthesised, positively charged antimicrobial peptides which are divided into four classes based on the chemical structure gene sequence similarities (Klaenhammer, 1993). They possess a narrow spectrum of activity, only targeting members of the same, or closely related species (Jack *et al.*, 1995). The inhibitory mechanisms of bacteriocins are varied and they target a multitude of intracellular processes (Yeaman & Yount, 2003). However, the positively charged nature of bacteriocins means they simultaneously attack the bacterial cell membrane as an inhibition mechanism or to gain access to the cellular targets (Yeaman & Yount, 2003). Changes in the charge on the bacterial cell surface can therefore result in broad resistance to a range of bacteriocins (Ernst & Peschel, 2011).

The ecological and evolutionary implications of bacteriocin production have been well studied. By only targeting closely related species and with a relatively high cost, bacteriocins production can be viewed as a spiteful trait (Gardner *et al.*, 2004). Models show that this spiteful behaviour is advantageous when the bacteriocin producer is at an intermediate frequency in a population. If the frequency of the producer is too low, the sensitive strains will share some of the benefits, of reduced bacterial densities and relatively higher resources. If the frequency of the producer is too high then the cost of production will not be out balanced by the reduction in fitness of competing sensitive strains (Gardner *et al.*, 2004). Furthermore, the model by Gardner *et al.* (2004) also predicts that spiteful behaviour will decrease parasite virulence. The predictions that bacteriocin production is favoured at intermediate frequencies and reduces parasite virulence has also been

shown empirically by studying *Photorhabdus* spp infection in caterpillars (Massey *et al.*, 2004) and *Pseudomonas aeruginosa* infection of wax moth larvae (Inglis *et al.*, 2009). The production of bacteriocins by populations at low relative frequencies has also been shown to be beneficial in a spatially structured medium (Chao & Levin, 1981). Bacteriocin producers were able to invade sensitive populations at low frequencies with a spatial structure, which was not the case in a mixed liquid medium.

Antibiotics are a diverse range of compounds produced by soil dwelling microorganisms, most notably the *Streptomyces* spp. (Weber *et al.*, 2003). Their therapeutic use in modern medicine clearly demonstrates their potential as agents of interference competition in nature. Empirical studies strongly support this, with densities of sensitive bacteria being reduced in the presence of antibiotic producing organisms (Rasool & Wimpenny, 1982; Turpin *et al.*, 1992; Wiener, 1996). Furthermore, *in vivo* production of antibiotics in the rhizosphere has been shown to increase the producers competitiveness by increasing their ability to occupy root nodules (Robleto *et al.*, 1997; Robleto *et al.*, 1998) while non antibiotic producers suffer a reduced fitness (Mazzola *et al.*, 1992; Pierson & Pierson, 1996). Within the context of the rhizosphere, antibiotic producers also protect the plants they inhabit from pathogens (Compant *et al.*, 2005; Raaijmakers *et al.*, 2002). Conversely, plants uninhabited by antibiotic producing microorganisms are at a much greater risk of succumbing to pathogen invasion (Keel *et al.*, 1992; Silosuh *et al.*, 1994; Thomashow & Weller, 1988).

The benefits conferred by resident antibiotic producing microorganisms are not unique to plants in the rhizosphere. The fungal garden acting as a food source for Attine ants is protected from parasites by antibiotic producing *Pseudonocardia* (Currie *et al.*, 2006; Little *et al.*, 2008). Antibiotic producing bacteria are also responsible for the protection of crustacean embryos (Gilturnes *et al.*, 1989; Gilturnes & Fenical, 1992), wasp larvae (Kaltenpoth *et al.*, 2005) and spruce bark beetles (Cardoza *et al.*, 2006) from parasitic infection.

While interference competition seems a likely reason for the production of antibiotics in the natural environment there are other explanations. In many instances, antibiotics are not detected from the soil where antibiotic producers are isolated (Linares *et al.*, 2006). In addition, antibiotics are known to affect the quorum sensing systems of the organisms which produce them, perhaps indicating that their purpose is for signaling between bacterial cells (Davies, 2006; Linares *et al.*, 2006; Martinez, 2008; Yim *et al.*, 2006). It has also been shown that some microorganisms can subsist entirely on antibiotics, using them as their sole carbon source (D'Costa *et al.*, 2006; D'Costa *et al.*, 2007; Dantas *et al.*, 2008; Martinez, 2008; Wright, 2007).

Toxic secondary metabolites are perhaps the most difficult to identify as sources of interference competition as most can be toxic at high enough concentrations (Tagg *et al.*, 1976). However, they can cause interference competition under the right circumstance. Some of the most widely studied toxic chemicals are those produced by cyanobacteria which when produced

in blooms are not only toxic to algae in the local environment competing for nutrients (Leão *et al.*, 2009), but also to fish in the proximity (Dong *et al.*, 2011; Guroy *et al.*, 2011), humans (Testai & Funari, 2010) and livestock (Collett *et al.*, 2011) which drink the water. *Streptococcus pneumoniae* is also known for the production of hydrogen peroxide, which was proposed to be an agent of interference competition, specifically against *S. aureus*. It seems unlikely that *S. pneumoniae* produces inhibitory levels of hydrogen peroxide to act directly, however Selva *et. al.* (2009) demonstrated a novel mechanism of interference by which the sub inhibitory levels of hydrogen peroxide reduce the population density of *S. aureus* by inducing temperate phage.

Interference competition affects many community processes by altering the relative fitness of one organism compared to another, giving a competitive advantage either by increasing the producers' fitness, or more likely significantly reducing the fitness of competitors. Invasion is one of the best studied ecological phenomena with respect to interference competition. Production of allelopathic compounds were shown to significantly increase the invasion success of bacterial producers in a spatially structured environment (Chao & Levin, 1981).

4.2 Aims

The aim this chapter is to elucidate interference competition interactions between nasal isolates and *S. aureus* and to determine if these interactions drive community composition. Despite the described weakness for community sampling the main strength of our culture-based sampling

approach is that the individual isolates can be assayed for their ability to inhibit *S. aureus*. This antagonism assay also lends itself to a relatively high throughput screen format, as well as potentially enabling inferences about interactions in the niche. These data can then be applied to the meta-community analyses and the contribution of interference competition to driving community structure can be assessed.

4.3 Materials and Methods

4.3.1 Interference competition spray assay

A 25 µl spot (approximately 10^8 cells) of an overnight bacterial culture (method 2.1) of each nasal isolate was pipetted onto the centre of an agar plate containing 15 ml of BHI agar (lab M). The plates were incubated for 18 h at 37°C before 250 µl of a ten-fold diluted overnight culture of *S. aureus* SH1000 (10^6 cfu) was sprayed over the plate. The plates were incubated for a further 18 h after when the size of the inhibition zone produced by the central nasal isolate on SH1000 was assessed. The clarity of the inhibition zone was scored based on a simple scoring system of 1 to 4, 4 being completely clear and 1 being no detectable zone. The areas of any detectable zones were also recorded by measuring the diameter of the inhibition zone and the central colony. The area of both the zone and the colony were calculated using the equation $A = \pi(d/2)^2$ where d is the diameter of the colony or the inhibition zone. The central colony area was then subtracted from the total zone area, leaving only the area of the zone around the perimeter of the central colony.

4.4 Results

In chapter one, several taxa were found to be negatively associated with *S. aureus* across the human nasal meta-population. A logical next step was to determine if there were physiological interactions between these taxa and *S. aureus* that could explain the observed distribution. Direct antagonism was selected primarily as an easily measurable bacterial trait that could infer real interactions in the microbial niche. The ability of every bacterial isolate to inhibit the *S. aureus* lab strain SH1000 was tested using the spray assay (Method 4.3.1). The zones of inhibition resulting from the spray assay were scored for clarity (1 – 4; 4 being completely clear and 1 being no detectable zone) (Fig. 4.1) and their area was calculated. The median zone clarity score for each taxon is shown in figure 4.2.A. The median was chosen since clarity score is a discrete variable and is non-normally distributed. Taxa present at higher regional frequencies tended to exhibit high variance in clearing scores between isolates. By contrast, taxa present at very low regional frequency exhibited low variance of the clarity scores as the sample size for these taxa were so small. The mean area of the inhibition zone for each taxon is shown in Figure 4.2.B. Four taxa (PRO, MAC, SCH and GHA) displayed no inhibition, and yet were found to be significantly negatively associated with *S. aureus* (Table 3.2). This supports the conclusion that direct antagonism is not the mechanism causing this exclusion. All the other taxa found to be negatively associated with *S. aureus* (Fig. 3.2), produce varying sizes of inhibition zone, although it is not clear from this species-level data whether inhibition is directly responsible for the exclusion.

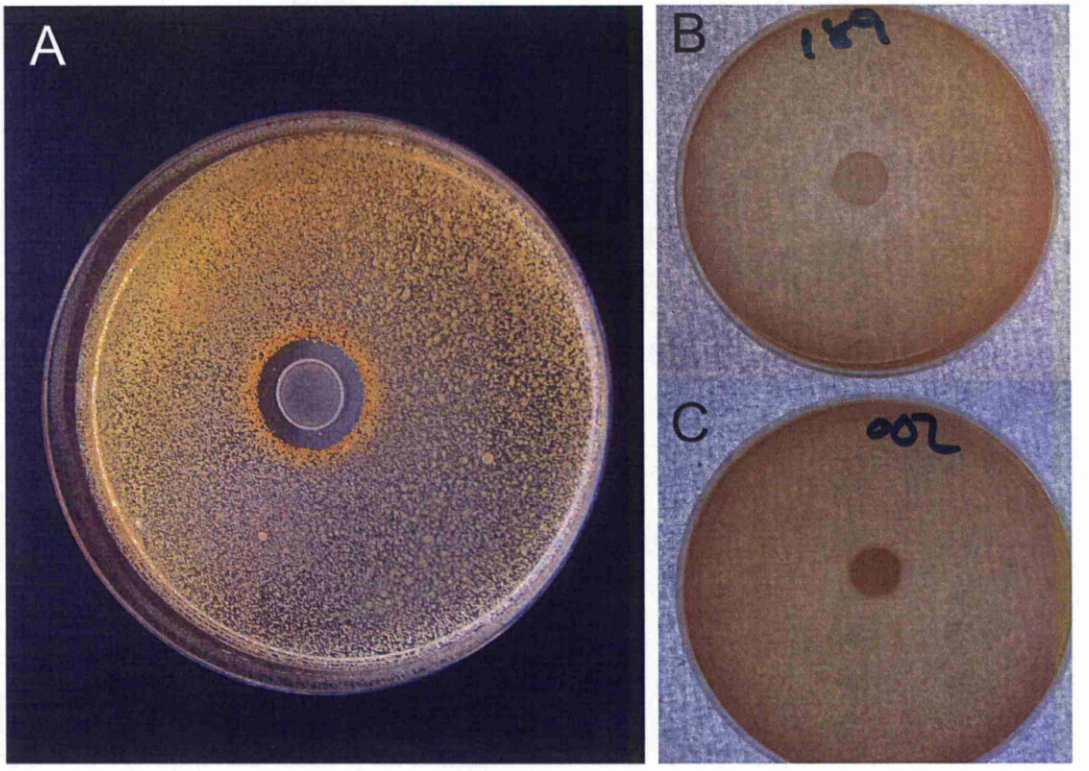


Figure 4.1. Gradient of SH1000 lawn growth (1 – 4). No growth (4) to normal growth (1). Panel A shows no growth (4), panel B shows cloudy growth (3) panel C shows non normal growth but no apparent inhibition (2) and normal growth (1) is not shown.

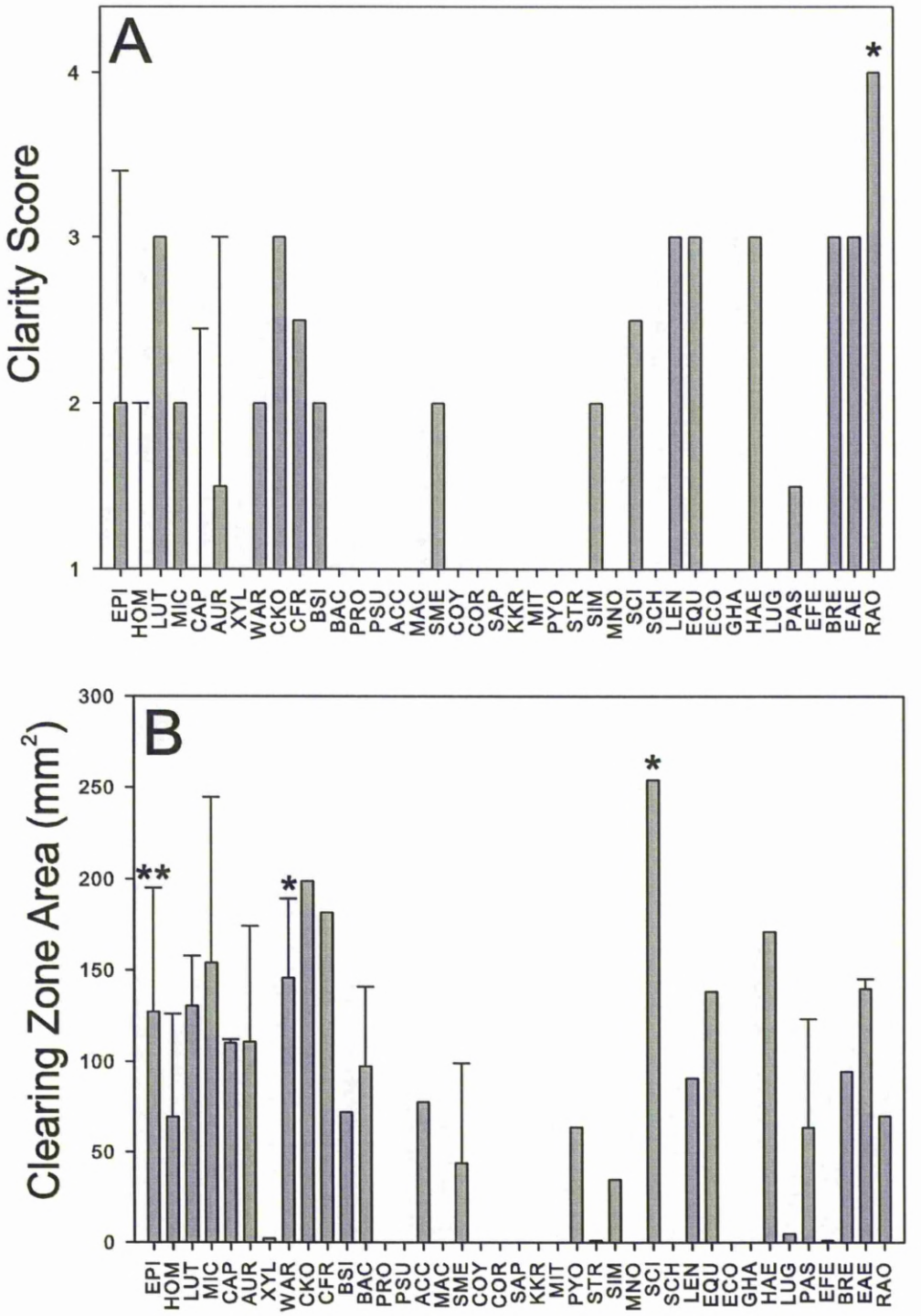


Figure 4.2. Clarity scores associated with each taxon. (A) Median clarity score for each taxon as the scores are discrete variables and are not normally distributed, the error bars represent the 90th percentile limit. Asterisks represent a significant difference from one (i.e. no clearing zone) using a Behrens-Fisher test. B shows the mean area or the zone for each taxon, error bars represent 1 standard error. Asterisks represent a significant difference from zero using a *post hoc* Dunnett's test. One asterisk signifies $p<0.05$, two asterisks show $p<0.01$.

To determine whether inhibition phenotype of isolates had an effect on the distribution of *S. aureus*, the presence or absence of taxa in each community weighted by inhibition was plotted using heatmaps (Fig. 4.3). Figure 4.3.A shows the taxa distribution with the colours overlaid representing the inhibition zone clarity and Figure 4.3.B shows taxa distributions with inhibition zone area overlaid in colour (red being most inhibitory and green being non inhibitory). Figure 4.3.A supports the existence of a negative relationship between clarity score and *S. aureus* presence or absence, with the majority of the inhibitory (red and orange) isolates being present in communities where *S. aureus* is absent. This is most clear in the case of *Micrococcus luteus* (LUT), which comprises of three inhibitory isolates, all of which do not co-exist with *S. aureus,* and three non-inhibitory isolates which do co-exist with *S. aureus.* A similar pattern is seen with the distribution heatmap overlayed with the inhibition zone area (Fig. 4.3.B), with most of the inhibitory *S. epidermidis* (EPI) and *Micrococcus luteus* (LUT) not co-existing with *S. aureus.*

A GLM with a logit link function was used to ascribe statistical significance to these observations. The presence or absence data (Table 3.2) was weighted with the inhibition zone clarity data (Table 4.1.A) and a second GLM was weighted with the inhibition zone area (Table 4.1.B). *S. epidermidis* (EPI), *S. hominis* (HOM) and *Micrococcus luteus* (LUT) were significantly negatively associated with *S. aureus* in both models. Antagonism may therefore explain the observed negative association between *S. aureus* and *S. epidermidis* already highlighted in Table 3.2. Two other taxa to which this might also

apply are *S. capitis* (CAP) and *C. propinquum* (PRO), which were both shown to be significantly negatively associated with *S. aureus* when weighted with the inhibition clarity scores. However, the absence of *C. propinquum* is not likely to be explained by inhibition zone using the assay described here since it does not show any inhibitory activity, but as shown in the model (Fig.. 4.3) as it only occurs in two communities, neither of which contain *S. aureus.*

Interestingly, by weighting the taxa by their inhibition potential, completely novel interactions have been uncovered indicating that only inhibitory strains within the taxa groupings are negatively associated with *S. aureus.* These novel associations include *Micrococcus luteus* (MIC) and *S. hominis* (HOM), as well as *Citrobacter freundii* (CFR) and *Corynebacterium* sp. (COR).

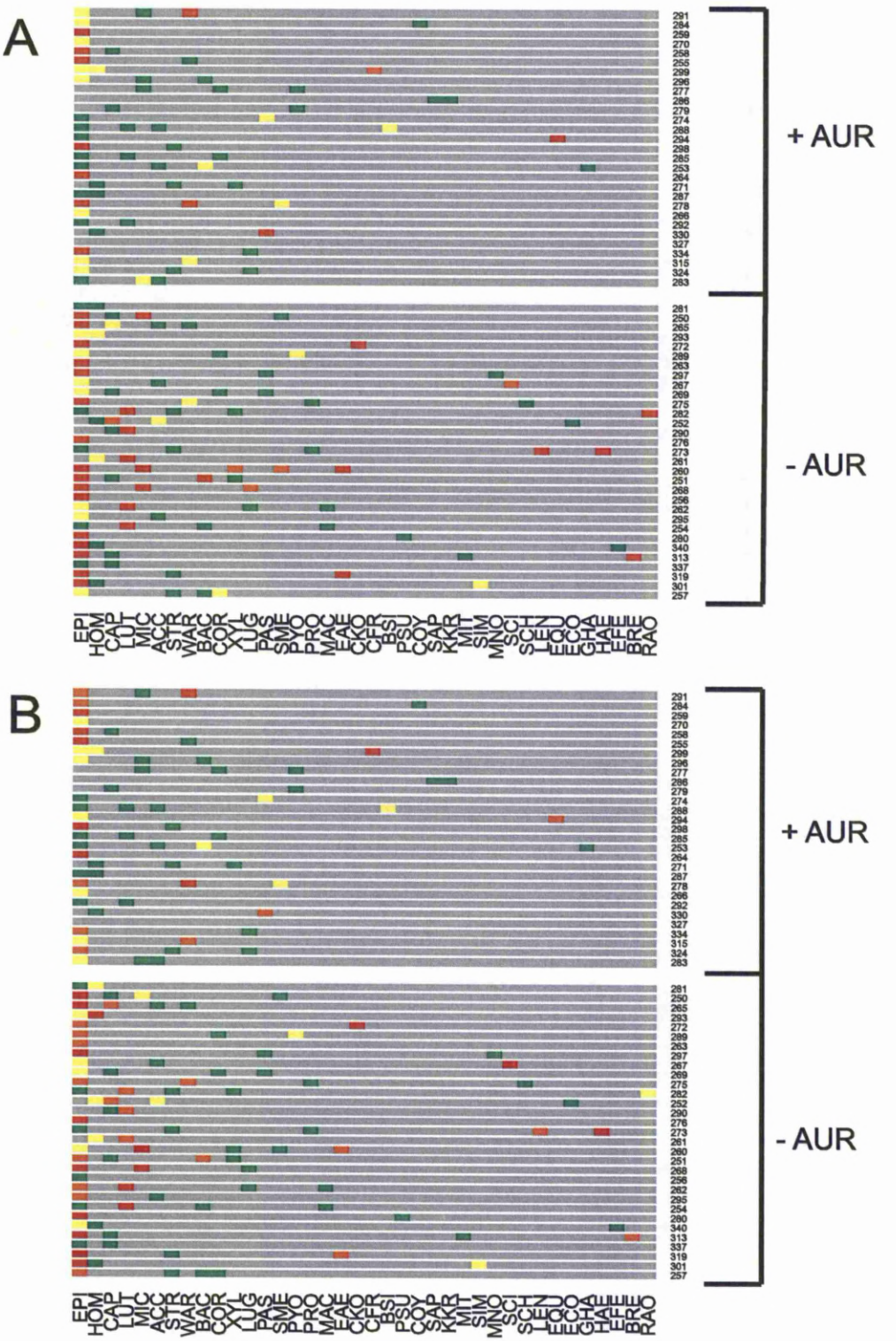


Figure 4.3. Taxa distribution with overlaid inhibition data. (A) Taxa distribution overlaid colours indicating the clearing zone score. (B) Same distribution with the colours representing clearing zone area. In both cases the scale runs from green – no inhibition to red – maximum inhibition. Each heatmap is divided into communities containing *S. aureus* (+AUR) and communities not containing *S. aureus* (-AUR).

	Df	Deviance	AIC	LRT	COR	Pr(Chi)
†EPI	1	50.33935441	76.33935441	16.9934966	-0.29	***3.75E-05
HOM	1	44.68477221	70.68477221	11.3389144	-0.08	***0.000758997
LUT	1	50.81713148	76.81713148	17.47127367	-0.23	***2.92E-05
†CAP	1	41.92128512	67.92128512	8.575427304	-0.26	**0.0034073
CKO	1	35.72926185	61.72926185	2.383404041	-0.13	0.122630073
CFR	1	39.32410478	65.32410478	5.978246966	0.13	*0.014483391
BSI	1	35.73997032	61.73997032	2.394112505	0.13	0.121792852
†PRO	1	43.48209536	69.48209536	10.13623755	-0.18	**0.001453826
PSU	1	35.72926185	61.72926185	2.383404041	-0.13	0.122630073
†ACC	1	39.93320763	65.93320763	6.587349823	-0.07	*0.010270595
COR	1	40.29812593	66.29812593	6.952268116	-0.09	**0.008371303
MNO	1	35.72926185	61.72926185	2.383404041	-0.13	0.122630073
†EAE	1	36.19846875	62.19846875	2.852610942	-0.18	0.091225674

Table 4.1.A

	Df	Deviance	AIC	LRT	COR	Pr(Chi)
†EPI	1	53.46719026	75.46719026	9.159165123	-0.22	**0.002474754
HOM	1	54.03626965	76.03626965	9.728244518	-0.19	**0.001814577
LUT	1	56.94875146	78.94875146	12.64072633	-0.29	***0.000377433
†MIC	1	49.10884469	71.10884469	4.800819559	-0.20	*0.028446202
CKO	1	46.37209776	68.37209776	2.064072631	-0.13	0.150806644
CFR	1	47.68908709	69.68908709	3.381061955	0.13	0.065949549
PYO	1	46.65028808	68.65028808	2.342262947	-0.13	0.125906533
STR	1	46.36692677	68.36692677	2.058901639	-0.13	0.151319208
SCI	1	46.80769284	68.80769284	2.499667704	-0.13	0.113870322
LEN	1	48.3477896	70.3477896	4.039764471	-0.13	*0.044440024
EFE	1	47.58597402	69.58597402	3.277948888	-0.13	0.070216631

Table 4.1.B

Table 4.1. GLM results of taxa distributions weighted by inhibition. The distributions among all of the samples of each taxa was compared to the presence or absence of *S. aureus* using distribution data weighted by inhibition score (A) or by distribution data weighted by inhibition area (B). The likely hood ratio test (LRT) and p-values (Pr(Chi)) show the significance of the distributions along with the Akaike Information Criterion which demonstrates the suitability of this model. COR indicates whether the fit of the model was positive or negative. The taxa with are also significant in the presence absence GLM (Table 3.2) are indicated with a dagger (†).

Determining the inhibitory phenotypes of the taxa has shed light on some completely novel interactions occurring between the microbial residents of the human anterior nares. It is also interesting to consider whether the level of inhibition in a community, regardless of the taxa present, contributes to *S. aureus* distribution across the meta-community. To test this the sum inhibitory values of each community was determined. The sum of inhibition clarity score plotted against presence or absence of *S. aureus* is shown in Figure 4.4.A and the sum clearing zone area is shown in Figure 4.4.B. There was a significant negative relationship between presence of *S. aureus* and sum of inhibition for both inhibition zone clarity ($X^2_{22.44}$, Dev = 82.13, $p > 0.001$) and clearing zone area ($X^2_{5.44}$, Dev = 65.14, $p = 0.0196$). The fit for the inhibition zone area is not highly significant and it would be difficult to predict anything from the fit of the regression line. However, the fit for the logistic regression of cumulative inhibition zone clarity against *S. aureus* presence/absence is highly significant ($p = 2.17 \times 10^{-6}$). The regression line in Figure 4.4.A shows that there is just as much chance of encountering *S. aureus* in a community with a cumulative clearing score of 4, as there is of finding *S. aureus* in a community with no inhibitory taxa (just under an 80% chance). However, in communities with a clearing score of >5, the probability of encountering *S.aureus* is greatly reduced and rapidly approaches 0%. In real terms, this means that *S. aureus* can co-inhabit a niche with one inhibitory organism with a score of 3 or 4, but if there is more than one inhibitory organism, the chance of finding *S. aureus* in that community is greatly reduced.

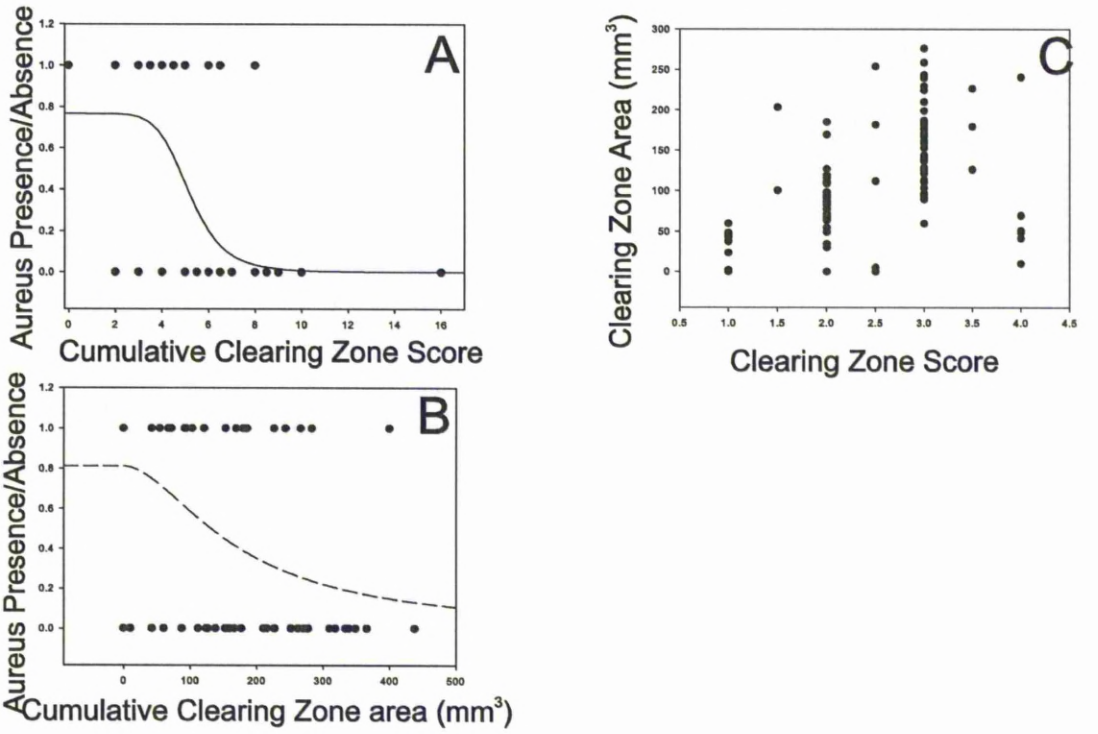


Figure 4.4. Community level inhibition attributes. A shows the a logistic regression curve fitted against the cumulative community inhibition score against the presence / absence of *S. aureus* ($X^2_{22.44}$, Dev = 82.13, p > 0.001). B shows a logistic regression curve fitted to the cumulative community inhibition zone area against the presence / absence of *S. aureus* ($X^2_{5.44}$, Dev = 65.14, p = 0.0196). C shows how the clearing zone score correlates with the clearing zone size.

4.5 Discussion

The results from this chapter clearly demonstrate that antagonism is a potential driver of the microbial community structure in the human nose and validates the use of culture dependent methods in the study of microbial ecology. Some taxa previously found (chapter 3 - Table 3.2) to be negatively associated with *S. aureus* were shown to more strongly negatively associated when weighted by inhibitory phenotype (GLM, Table 4.1, EPI, CAP, PRO, ACC and MIC). One taxon (EAE) was shown to be significantly negatively associated when only considering the presence and absence of species data, however when the inhibition data were overlaid, the association was no longer significant, indicating that the negative association was caused by another mechanism. Furthermore, several taxa were only found to be negatively associated with *S. aureus* when inhibition data was used as a weighting in the GLM, strongly suggesting that inhibition plays a role in their negative association (HOM, LUT, CFR, COR and LEN) (Fig. 4.3). This study suggests that interspecific phenotypic variation is an important factor driving species distribution.

A major benefit of this study is that the interactions observed against SH1000 were not just from representative species being used to investigate a general phenomenon but from the exact species that were found and isolated from the sampled communities. As the amount of variation in the ability of an individual taxon to inhibit SH1000 is so great (most notable *S. epidermidis* Fig. 4.3.A & 4.3.B) using a representative of the species to find interactions relevant in the niche is essentially not possible. To truly find interactions

relevant to the niche, the niche from which the organisms came must be taken into account. By doing this, the results of the inhibition of natural isolates on SH1000 may explain some aspects of *S. aureus* distribution in the niche. Using natural isolates of *S. aureus* in the inhibition spray assay, may have more accurately explained *S. aureus* distribution. This was not done however, due to physical experimental constraints.

Perhaps the clearest example of the benefits of this approach is the discovery that inhibitory strains of *S. homins* (HOM) and *Micrococcus luteus* (LUT) were far less likely to co-occur with *S. aureus* than non-inhibitory strains of the same species. This observation could not have been made by only considering the presence and absence of species as in Table 3.2, and as is done in all of the culture independent methods to date. In this example, it is not simply the species that is important, but the phenotypes of the specific isolates, which may be highly polymorphic across a species.

The sum of inhibitory phenotypes at the community-level was also shown to be important. If a community contains a number of different taxa and the sum of their clearing zone scores is greater than 5, then there is very low probability that *S. aureus* will be found in this community, seemingly regardless of which taxa are present (Fig. 4.4.A). The same trend can be seen for the cumulative area of the clearing zone produced by the taxa in a community although the effect is not as strong (Fig. 4.4.B). The notion that if a community contains a large number of taxa antagonistic towards any given

species, then that species will be less likely to be found there seems obvious but it has not, to my knowledge, been empirically tested in this way.

The bacteriocidal nature of the interference competition was not determined as cell free activity of the compounds could not be detected. The only insight into the nature of the allelopathic molecules is the type of the zone they produce (Fig. 4.1) and the relationship between the area and clarity of the zone (Fig. 4.4.C). Figure 4.4.C shows that there is a negative relationship between the size of a cloudy zone (zone type 3) and the size of a clear zone (zone type 4) with the cloudy zones tending to have a larger area. This may suggest that the clearing zones are caused by diffusible molecules of different sizes. The smaller molecules diffuse further through the agar plates and become diluted, producing a cloudy zone whereas the larger molecules cannot diffuse as far, becoming concentrated around the colony and producing a much clearer zone. Unfortunately, as no cell free activity could be isolated from the media, this hypothesis was not tested. With cell free activity of the allelopathic component it would have been possible narrow down exactly what the component was by heat treating it, subjecting it to different proteases and even determining the sizes of the compounds using electrophoresis.

Culture dependent approaches currently have the upper hand when elucidating physiological interactions. However, the relatively new field of transcriptomics raises interesting new possibilities for the study of microbial interactions within a community (Güell *et al.*, 2011). At present it is possible

to detect which genes are expressed in a community and which organisms are present. This means that the community wide regulation of genes could be correlated to the presence or absence of certain organisms. It is not currently possible to segregate the genes that are regulated into different species, if their genes are highly conserved between species. An even simpler method is to use metacommunity data to determine if the presence of individual genes appear to exclude certain organisms. Based on the findings in this chapter that inhibitor-producing isolates are less likely to coexist with *S. aureus,* it would be interesting to investigate whether the presence of inhibitor-producing genes (bacteriocins, antibiotics, PSM's etc) in the metacommunity negatively associated with *S. aureus.* Furthermore, this could be a way to investigate resource competition in the niche, by identifying metabolic genes and pathways that also exclude *S. aureus.*

Species boundaries in bacteria are very difficult to define. However, this analysis suggests that it is arguably more important to consider the phenotypes that drive community dynamics, in parallel with identification of species. After all, it is not the species *S. aureus per se* that is important in an infection, but the many phenotypes it possesses capable of causing these infections. In the same way, the taxa present in a community may be less important in excluding *S. aureus* than the phenotypes present among the taxa in a community that cause the exclusion.

Chapter 5: Do biofilm disruption and Agr signaling interference contribute to the distribution of *S. aureus* across communities?

5. 1 Introduction

Interference competition mediated by the production of toxins that directly kill a competitor potentially affects species distribution among the nasal microbial communities (chapter 4). However, this form of direct antagonism is not the only mechanism by which microorganisms benefit by secreting secondary metabolites to reduce the fitness of competitors. These chemicals produced by microorganisms may appear to have no effect *in vitro* but in their natural environment potentially serve to reduce the relative fitness of competitors (Iwase *et al.*, 2010; Lina *et al.*, 2003). The major difficulty in uncovering these interactions is that an understanding of the factors that affect fitness and survival in the natural niche must first be determined and since these factors are often complex, assumptions are necessary. However, two mechanisms for this type of interference competition in bacteria have been well-studied: inhibition of biofilm formation (Iwase *et al.*, 2010; Quinn *et al.*, 2009) and the disruption of quorum sensing (Diggle *et al.*, 2007; Fleming *et al.*, 2006; Lina *et al.*, 2003).

Biofilm formation may be important for survival of *S. aureus* in the nasal niche (Iwase *et al.*, 2010; Quinn *et al.*, 2009). Studies have shown that strains of *S. epidermidis* possessing endoserine peptidase (ESP) activity are capable of inhibiting formation of the *S. aureus* biofilm *in vitro,* as well as

destroying preexisting biofilms. Furthermore, ESP-producing strains can displace established *S. aureus* populations from the anterior nares (Iwase *et al.*, 2010). Although the precise mechanism for ESP mediated biofilm disruption is not known, it is likely that it cleaves proteins involved in biofilm formation. Known biofilm-associated proteins include cell wall-associated proteins SasG and Pls (Corrigan *et al.*, 2007), the autolysin Atl (Biswas *et al.*, 2006; Heilmann *et al.*, 1997) and the biofilm-associated protein Bap, found only in bovine *S. aureus* strains, (Lasa & Penades, 2006). *Pseudomonas aeruginosa* secretes extracellular molecules that prevent biofilm formation by *S. epidermidis* as well as disrupting established biofilms (Pihl *et al.*, 2010; Qin *et al.*, 2009). This disruption was proposed to be mediated by an extracellular polysaccharide produced by *Ps. aeruginosa,* since cellulase treated supernatant, and *Ps. aeruginosa* mutants deficient in polysaccharide biosynthesis were not inhibitory. Inhibition is hypothesized to be caused by the interaction of the *Ps. aeruginosa* polysaccharide with the *S. epidermidis* polysaccharide that is important for biofilm formation (Qin *et al.*, 2009). Furthermore, small secreted molecules produced by *Ps. aeruginosa* were shown to inhibit biofilm formation by *Aspergillus fumigatus* (Mowat *et al.,* 2010). Beyond the nasal microbiome, competition via biofilm disruption has been well studied among dental microorganisms. *In vitro* prevention of *Streptococcus salivarius* formation by *Strep. mutans* was demonstrated when the organisms were co-cultured in 96 well plates. Inhibition was shown to be caused by disruption of the signaling pathway that stimulates biofilm formation in *Strep. mutans* (Tamura *et al.*, 2009). Urogenital biolfilms of harmful *E. coli* strains were significantly disrupted by extracellular products

from members of the normal vaginal flora, demonstrating that natural competition among microbes can prevent disease (McMillan *et al.*, 2011). This antagonism was probably caused by acid production, bacteriocins or surfactants that can penetrate and break up the biofilm (Cadieux *et al.*, 2009; Niku-Paavola *et al.*, 1999; Reid *et al.*, 1999; Silva *et al.*, 1987; Velraeds *et al.*, 1996).

Quorum sensing in *S. aureus* is mediated by an autoinducing peptide (AIP) that regulates a raft of cellular processes (Thoendel *et al.*, 2011). Interference of quorum sensing systems often results in organisms being unable to survive in the niche using infection models e.g. a *S. aureus* wound infection (Smith *et al.*, 2009); an *S. epidermidis* (Curtin & Donlan, *2006*) catheter biofilm and an *E. coli* biofilm on the vaginal mucosa (McMillan *et al.*, 2011). Several studies have demonstrated indirect interference competition against *S. aureus* via the quorum sensing system. This is caused by competing AIPs binding and blocking the surface sensor histidine kinase in the two component system (Ji *et al.*, 1997). Both intraspecific (Ji *et al.*, 1997) and interspecific inhibition by AIPs have been reported (Otto *et al.*, 1998), with the main interspecific interaction being with *S. epidermidis* AIPs *(Lina et al., 2003; Otto et al., 1998;* Otto *et al.,* 2001). Due to the high genetic diversity of both the AIP and the receptor (AgrC), it is difficult to predict the outcome of non-cognate AIP-receptor interactions (Dufour *et al.*, 2002). However, it is known that the macrocyclic ring of the AIP molecule can universally inhibit Agr signaling without the N-terminal tail (Lyon *et al.*, 2000). This suggests that inhibition will occur if the N-terminal tail of the AIP

molecule is not sufficiently complementary to the receptor as to elicit signal transduction (Lyon *et al.*, 2000). Agr interference *in vitro* was suggested by Lina *et al.* (Lina *et al.,* 2003) as a cause of *S. epidermidis* driven exclusion of *S. arueus* from the anterior nares. This exclusion was due to the fact that certain *S. aureus* and *S. epidermidis agr* types could not co-inhabit the same niche. Intraspecific Agr interference between clinical strains of *S. aureus* has also been shown to reduce virulence in an insect model (Fleming *et al.*, 2006).

Apparent interference competition can also occur when the competitive interaction is mediated by a third interacting biological entity (Bonsall & Hassell, 1997). The production of hydrogen peroxide by *Strep. pneumoniae* was previously thought to cause direct interference of *S. aureus*. A subsequent study contradicted this and stated that the H_2O_2 concentration produced by *Strep. pneumoniae* was not inhibitory to *S. aureus (*Regev-Yochay *et al.,* 2006). Moreover, recent work by Selva *et al.* (2009) empirically showed that sub-inhibitory concentrations of H_2O_2 produced by *S. pneumoniae* could significantly reduce the density of *S. aureus* by inducing the SOS response and triggering the induction of prophage harboured by the *S. aureus* population.

5. 2 Aims

The studies in this thesis have addressed direct, explicit interference competition in the form of excreted toxins. This chapter aims to investigate indirect interference competition by assaying selected nasal isolates for the ability to disrupt processes hypothesised to be essential for *S. aureus* survival in the niche, namely biofilm formation and Agr signal transduction.

5.3 Materials and Methods

5.3.1 Biofilm Inhibition Assay

Overnight cultures (method 2.1) of *S. aureus* strain SH1000 were inoculated with a 1% (v/v) inoculum into 96 well plates containing 50% single strength BHI supplemented with 4% (w/v) filter sterilised sucrose (final concentration 2% (w/v)) and 50% spent supernatant from a 18 h old culture of either *S. epidermidis, S. capitis* or *Corynebacterium* sp., up to a total volume of 200 µl. Supernatant from an 18 h culture of strain SH1000 and single strength BHI media were used as controls. Three biological replicates of the culture supernatants were used. The 96 well plates were incubated statically in a humidified environment for 48 h. Following incubation, the media was removed from the 96 well plates by pipetting, taking care not to disturb the biofilm on the base of the well. The biofilm was then washed 3 times by gently pipetting 200 µl of PBS over the biofilm and removing it by pipetting. The plates were air dried before 50 µl of 0.1% (w/v) crystal violet solution was added to each well and left for 10 minutes to stain the cells in the biofilm. The crystal violet solution was removed and the stained biofilm was washed by pipetting with PBS as previously described. The stained biofilm was then resuspended in 100 µl of 100% (v/v) glacial acetic acid and the optical density was read at 590 nm using a Victor 3 plate reader [Wallac].

5.3.2 Quorum sensing (Agr) interference

S. aureus strain Liv985 (SH1000 pSB2035 [*agr::luxABCDE]*)), which contains the *luxABCDE* genes under the control of the *agr RNAIII* P3 promoter was used to examine Agr signaling interference. Strain Liv985 was incubated in

96 well plates with 50% (v/v) single strength BHI and 50% (v/v) culture supernatant from a test strain. Culture supernatants from *Enterococcus feacalis* (L058 – Liverpool Strain) and *Bacillus cereus* (L001 – Liverpool Strain) were used as negative controls are not known to produce similar AIPs to *S. aureus* and are unlikely to interfere with Agr signal transduction. *E. faecalis* utilises pheromones which are structurally distinct from the staphylococcal AIP (Nakayama *et al.*, 2001a) and *B. cereus* relies on the competence system, not agr for cell density sensing (Kramer *et al.*, 2007). Single strength BHI media was also included as a control. Optical density readings (A_{590nm}) and relative luminescence units per second (RLU s^{-1}) were recorded after 7 h when luminescence and thus transcription from *agr* P3 were found to be highest.

5.3.3 Statistical Analysis

Behrens Fisher Multiple comparsions

Behrens Fisher multiple comparison were used to compare several means to the selected control value. The test was controlled for type II errors (alpha = 0.05). A conservative control (i.e. the lowest value) was always chosen for comparison when looking for either biofilm inhibition or Agr interference. The test was therefore constrained to only identify strains with significantly lower means than the control. Higher means could potentially be false positives, as the controls for comparison were selected for their conservatively low value. The analysis was done using the "npmc" package in R (R Development Core Team, 2010).

Logistic Regression

Logistic regression was carried out using a GLM with a logit link function (Method 2.23).

5.4 Results

5.4.1 Effect of isolate on biofilm production

Three candidate taxa were assayed for their ability to inhibit *S. aureus* biofilm formation and to disrupt Agr signal transduction. The taxa *S. epidermidis, S. capitis* and *Corynebacterium* spp. were chosen because of their observed negative associations with *S. aureus* (chapter 3). *S. epidermidis* has been previously reported to inhibit *S. aureus* biofilm formation (Iwase *et al.*, 2010) and interfere with Agr signaling (Lina *et al.*, 2003). To date no mechanisms have been described to account for the negative associations observed between *S. aureus* and *Corynebacterium* spp. or *S. capitis*.

To examine biofilm inhibition, *S. aureus* strain SH1000 was incubated for 48 h in media containing 50% (v/v) culture supernatant from either *S. epidermidis, Corynebacterium* spp. or *S. capitis.* The quantity of biofilm produced after incubation was then measured after staining with 0.1% (v/v) crystal violet solution. Two controls were used in the biofilm inhibition assay, one labeled "media" contained fresh media and no culture supernatant and one labeled "SH1000" contained 50% culture supernatant from an *S. aureus* SH1000 culture. The bar graphs in Figures 5.1-5.3 present the optical density of the crystal violet which is proportional to the quantity of *S. aureus* biofilm. Logistic regression (second plot in Figs. 5.1 - 5.3) was used to determine if

there was a relationship between the ability of a strain to inhibit biofilm formation and whether it was isolated from a microbial community containing *S. aureus*. All of the isolates were compared to the most conservative control by means of a Behrens Fisher nonparametric multiple comparison. The most conservative control (the lowest mean values) was selected for comparison to reduce the chance of false positive result.

The majority of *S. epidermidis* (37 out of 45) isolates significantly reduced the *S. aureus* biofilm when compared to the control (Fig. 5.1). However, there was no significant relationship between the degree of biofilm inhibition and the likelihood of coexistence with *S. aureus* in sampled communities (Table 5.1 & Fig. 5.1). None of the 21 *Corynebacterium* spp. isolates reduced the biofilm below the level of the media control (Fig. 5.2) (GLM $p = 0.8061$). Only 2 out of 10 strains of *S. capitis* (B098 & B160) significantly reduced the *S. aureus* strain SH1000 biofilm (Fig. 5.3) although these strains are not negatively associated with the presence of *S. aureus* in the niche (GLM $p = 0.6902$).

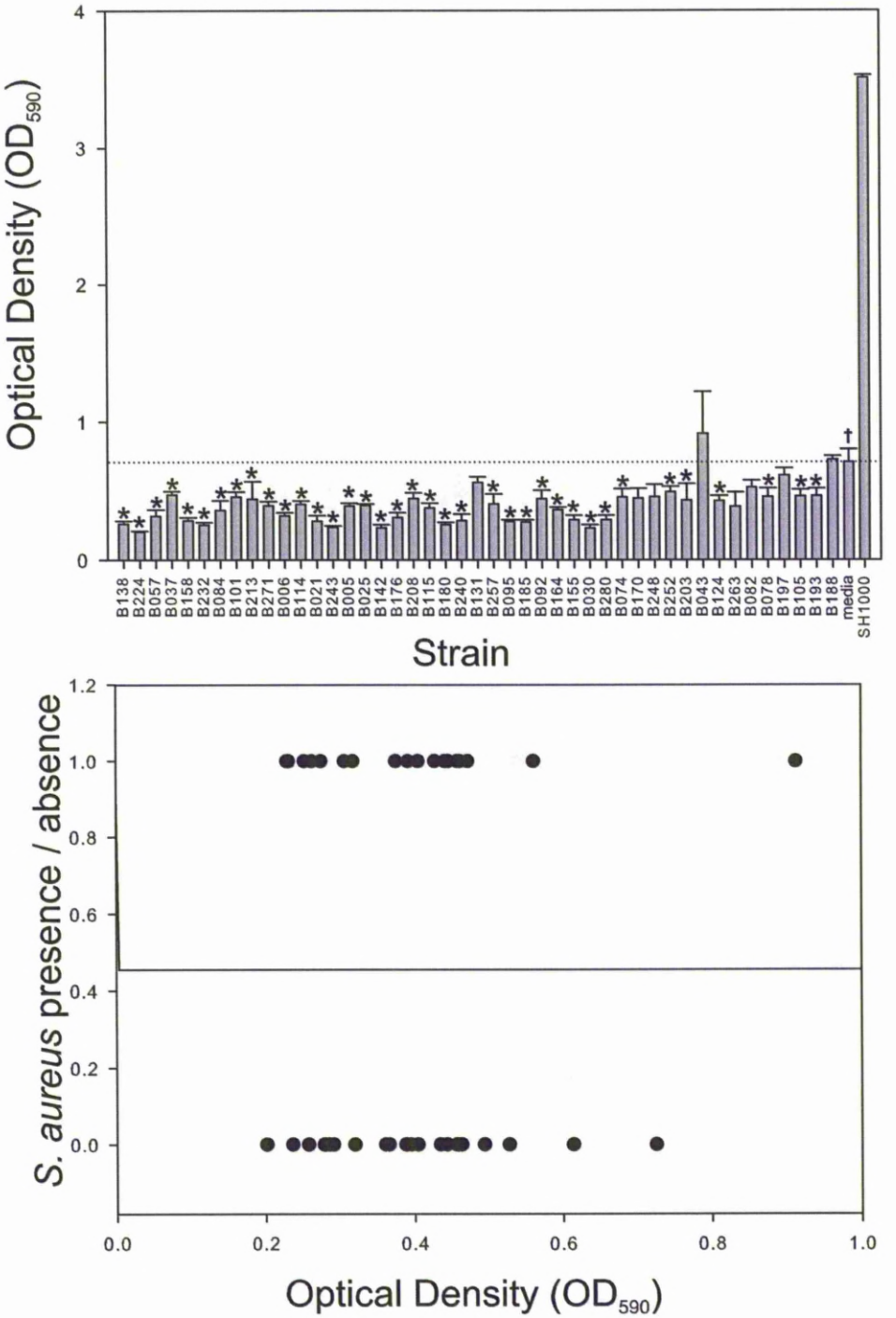


Figure 5.1. Effect of *S. epidermidis* on biofilm formation. The bar chart shows the quantity of *S. aureus* biofilm (OD of crystal violet stained and resuspended biofilm) able to grow in the presence of 50% supernatant present from each strain on the x axis. The dagger symbol indicates the control used for the multiple comparison (Behrens Fisher Test), and the dotted line shows the mean values of the control as a visual comparison. The second plot shows the relationship between the biofilm quantity measure in optical density and the presence of *S. aureus*. There is no significant relationship (GLM p = 0.6724).

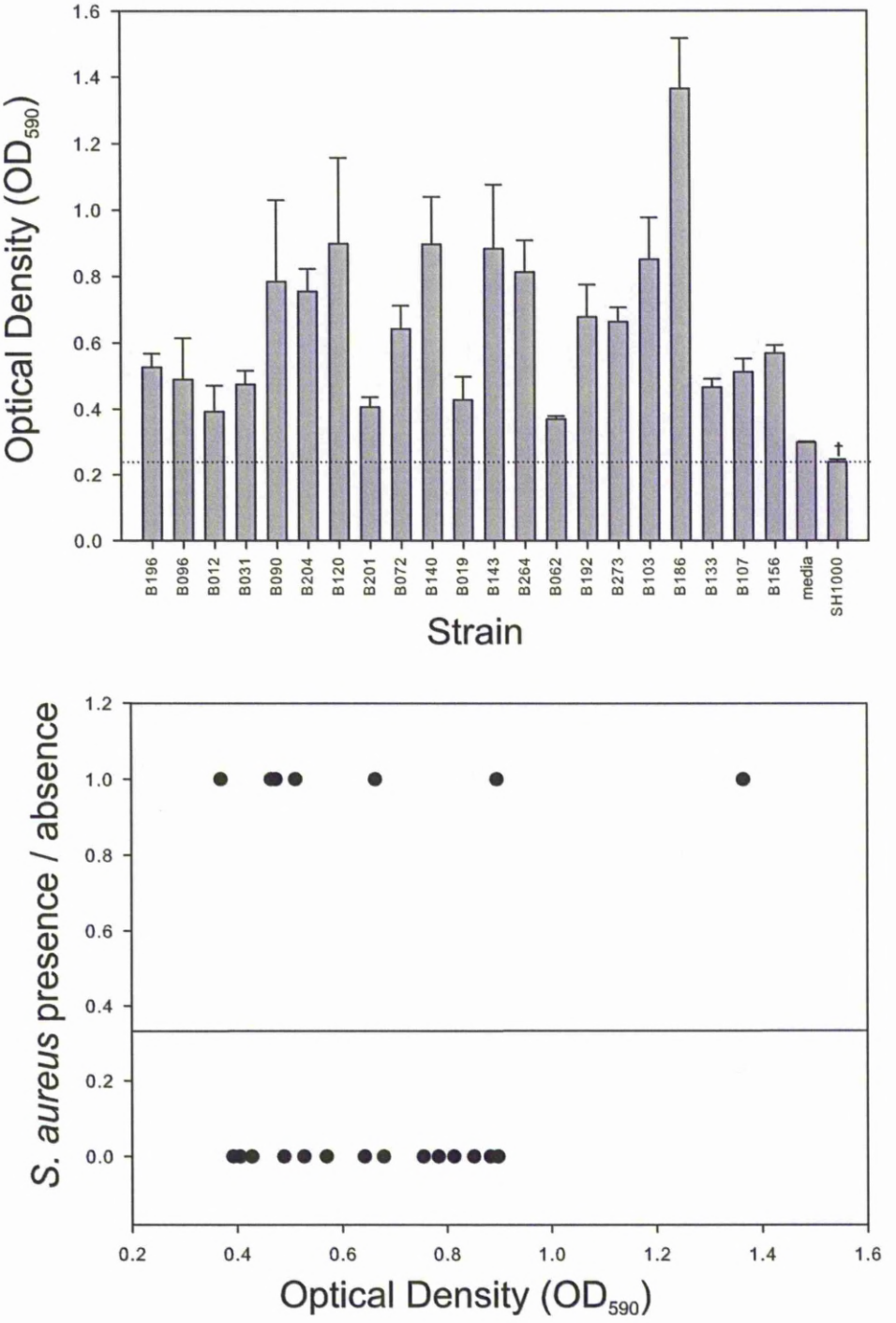


Figure 5.2. Effect of *Corynebacterium* spp. on biofilm formation. The bar chart shows the quantity of *S. aureus* biofilm (OD of crystal violet stained and resuspended biofilm) able to grow in the presence of 50% supernatant present from each strain on the x axis. The dagger symbol indicates the control used for the multiple comparison (Behrens Fisher Test), and the dotted line shows the mean values of the control as a visual comparison. The second plot shows the relationship between the biofilm quantity measure in optical density and the presence of *S. aureus*. There is no significant relationship (GLM p = 0.8061).

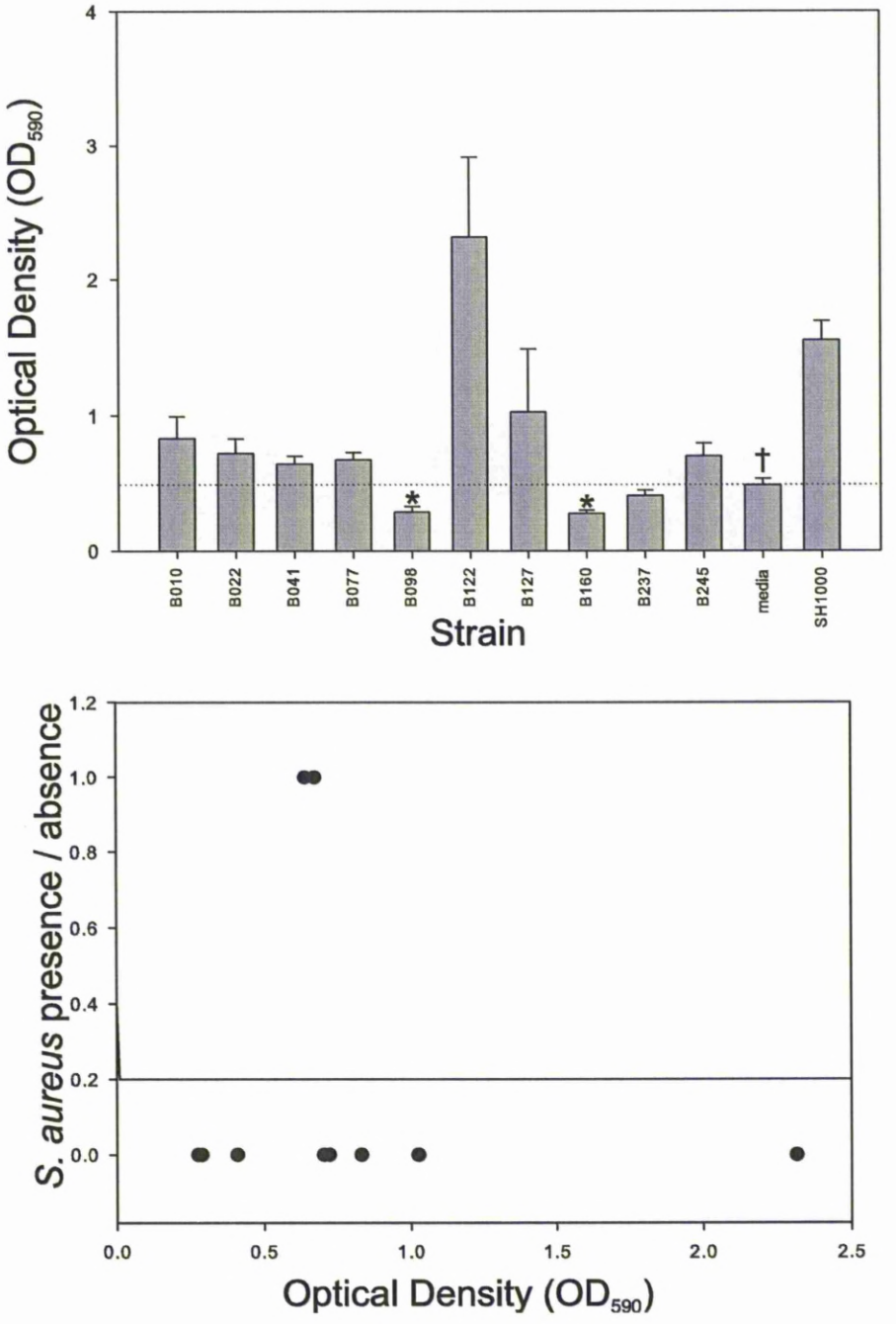


Figure 5.3. Effect of *S. capitis* on biofilm formation. The bar chart shows the quantity of *S. aureus* biofilm (OD of crystal violet stained and resuspended biofilm) able to grow in the presence of 50% supernatant present from each strain on the x axis. The dagger symbol indicates the control used for the multiple comparison (Behrens Fisher Test), and the dotted line shows the mean values of the control as a visual comparison. The second plot shows the relationship between the biofilm quantity measure in optical density and the presence of *S. aureus*. There is no significant relationship (GLM p = 0.6902).

5.4.2 Effect of isolates on agr transcription

To determine the effects of the isolates on *agr* transcription, a strain containing a plasmid with bioluminescence genes under the control of the *agr* promoter P3 was used. The Liv985 (SH1000 pSB2035 [*agr::luxABCDE]*) luminescent reporter plasmid strain was incubated in 50% culture supernatant (18 h) from each isolate. For controls, SH1000 culture supernatant and media were used as before. In addition, two other controls were selected: 50% supernatant from cultures of *Enterococcus faecalis* (L058) and *Bacillus cereus* (L001). These bacteria were chosen as they would deplete nutrients from the media and yet show no known evidence of *S. aureus* AIP mediated interference. As previously, comparisons were always performed against whichever of the two controls produced the lowest luminescence value for the most conservative analysis.

All except four of the *S. epidermidis* strains significantly reduced the RLU s^{-1} when compared to the L058 control (Fig. 5.4). There is also a significant negative relationship between RLU s^{-1} and presence of *S. aureus* as shown in Figure 5.4 and Table 5.2 (GLM – LTR = 4.039, p = 0.04446) indicating that *S. epidermidis* strains that inhibit *agr* transcription are more likely to be found in *S. aureus* containing communities. There was no correlation between biofilm inhibition and reduction in *agr* transcription (Spearman's R = -0.083, p = 0.59).

No *Corynebacterium* spp. isolates inhibited transcription of *agr* (Fig. 5.5) and there was no relationship between the levels of *agr* transcription and coexistence with *S. aureus* (Table 5.2 & figure 5.5).

All but one of the ten *S. capitis* strains were capable of significantly reducing *agr* transcription in *S. aureus* (Fig. 5.6). Furthermore there was a positive relationship between luminescence and the presence of *S. aureus* (Table 5.2), meaning that strains showing the greatest inhibition of *agr* transcription were least likely to be present in *S. aureus* containing communities (Fig. 5.6). The association was not significant ($p = 0.08674$) although this may have been caused to the relatively low numbers (number) of *S. capitis* isolates.

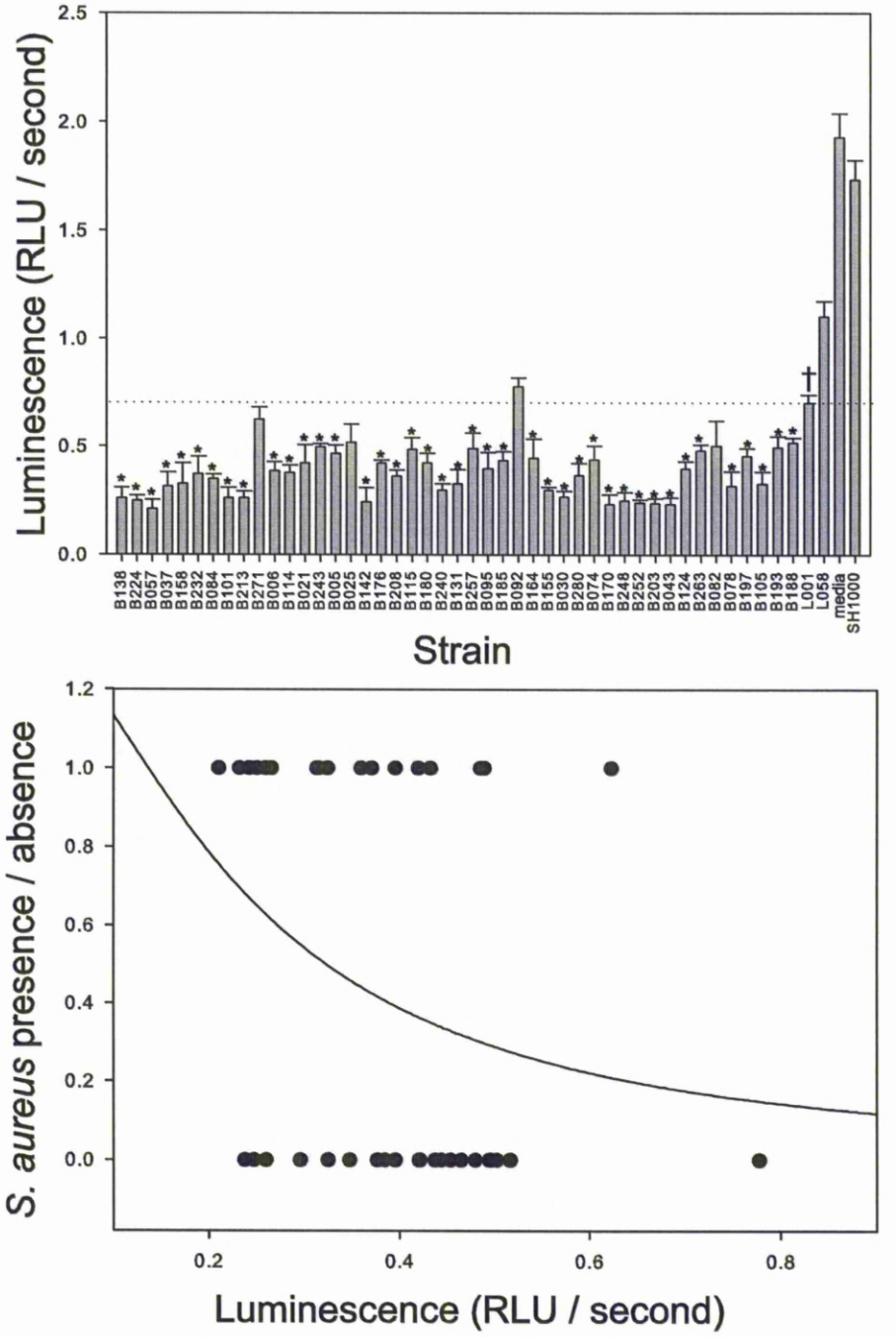


Figure 5.4. Effect of *S. epidermidis* on *agr* transcription per cell. The bar chart shows the *agr* transcription levels after 7 h in the presence of 50% supernatant present from each strain on the x axis. The dagger symbol (†) indicates the control used for the multiple comparison (Behrens Fisher Test), and the dotted line shows the mean values of the control as a visual comparison. The second plot shows the relationship between agr transcription levels measured in luminescence counts per second per optical density unit (CPS / od and the presence of *S. aureus*. There is a significant relationship between them (GLM p = 0.04446).

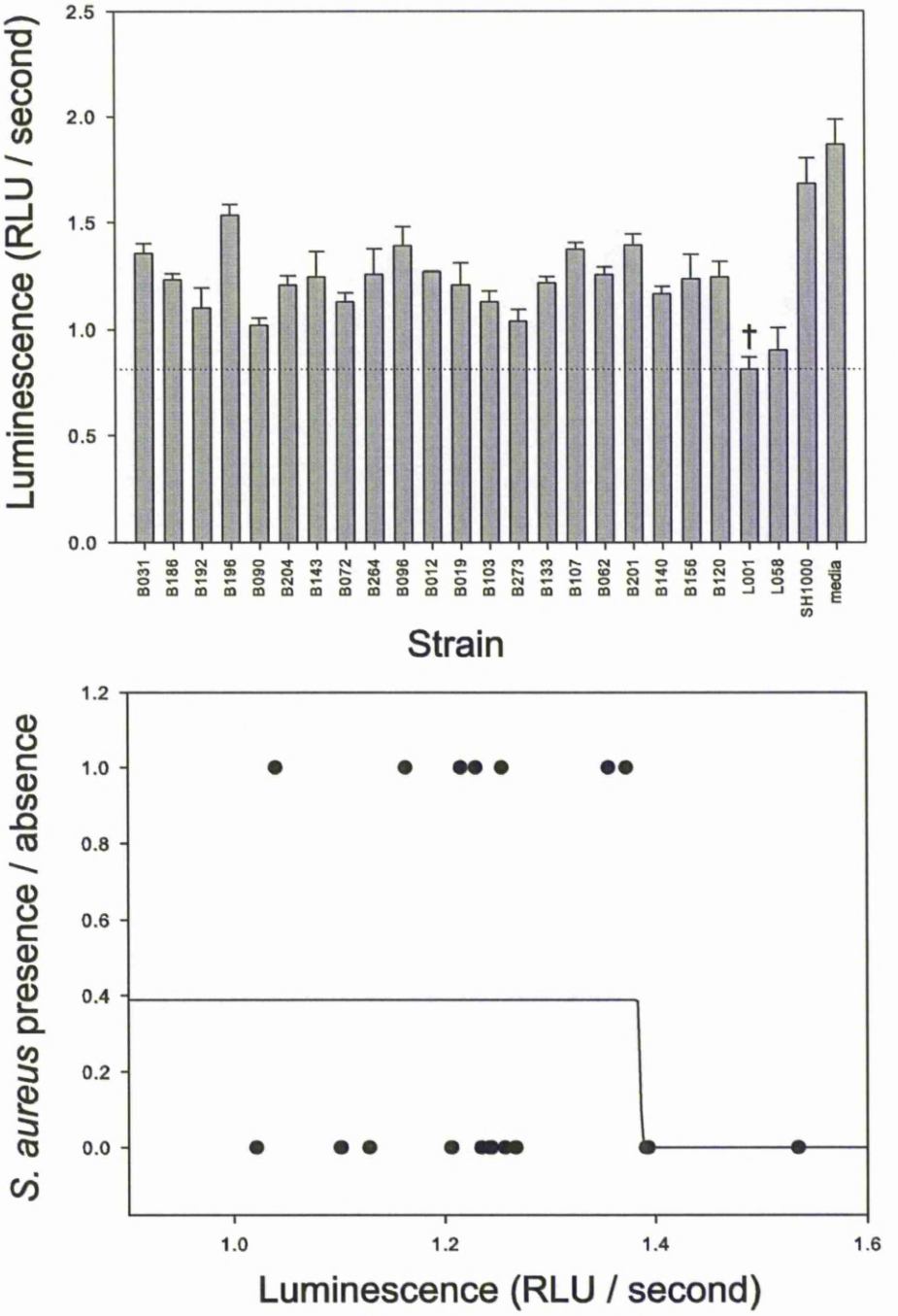


Figure 5.5. Effect of *Corynebacterium* spp. on *agr* transcription per cell. The bar chart shows the *agr* transcription levels after 7 h in the presence of 50% supernatant present from each strain on the x axis. The dagger symbol (†) indicates the control used for the multiple comparison (Behrens Fisher Test), and the dotted line shows the mean values of the control as a visual comparison The second plot shows the relationship between *agr* transcription levels measured in luminescence counts per second per optical density unit (CPS / OD and the presence of *S. aureus*. There is no significant relationship (GLM p = 0.9054).

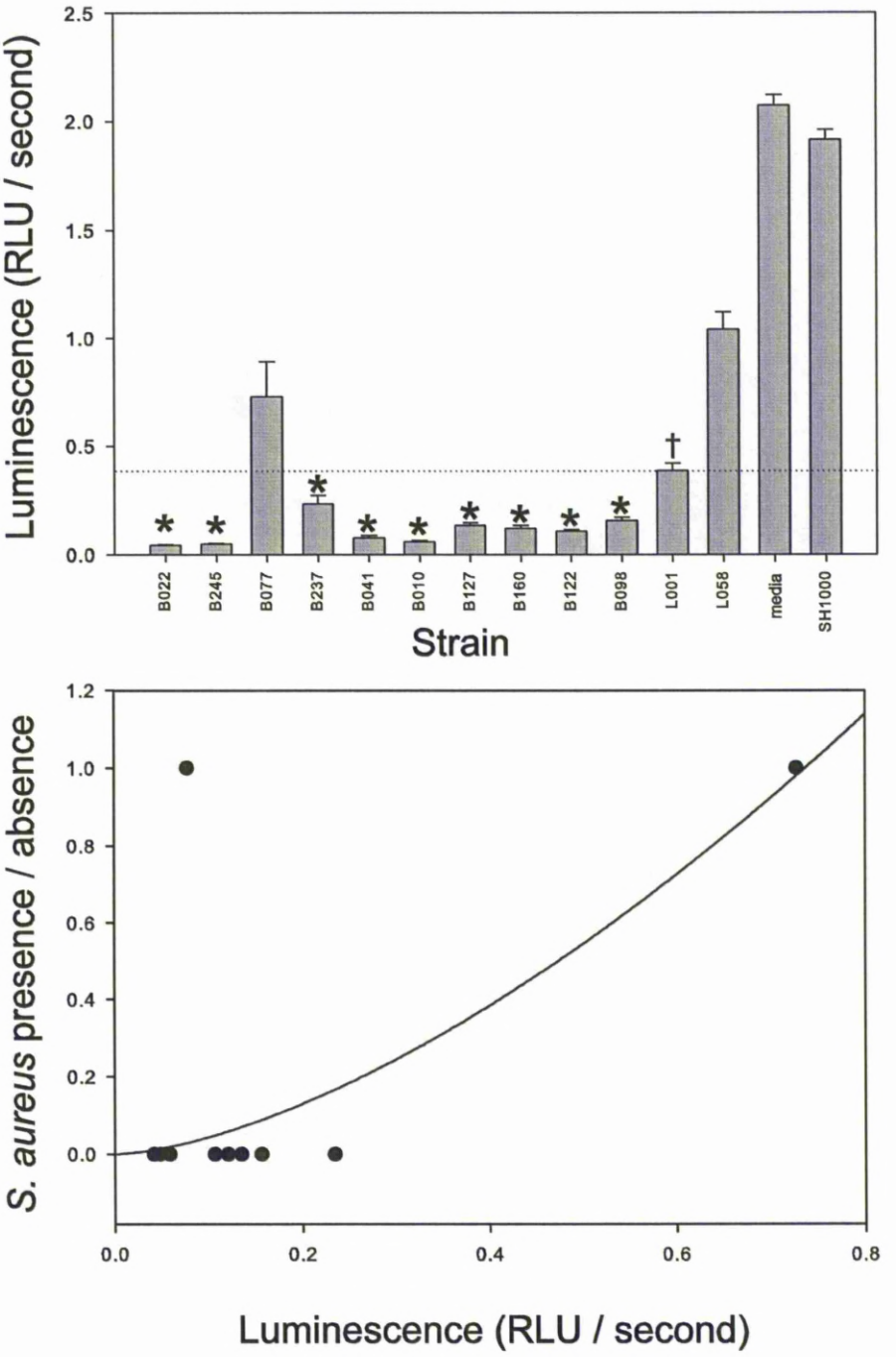


Figure 5.6. Effect of *S. capitis* on *agr* transcription per cell. The bar chart shows the *agr* transcription levels after 7 h in the presence of 50% supernatant present from each strain on the x axis. The dagger symbol indicates the control used for the multiple comparison (Behrens Fisher Test), and the dotted line shows the mean values of the control as a visual comparison. The second plot shows the relationship between *agr* transcription levels measured in luminescence counts per second per optical density unit (CPS / od and the presence of *S. aureus*. There is a significant relationship between them (GLM p = 0.08674).

	Df	Deviance	LRT	P -value
EPI	1	60.633	0.17880	0.6724
COR	1	26.734	0.060239	0.8061
CAP	1	10.0080	0.1589	0.6902

Table 5.1 GLM showing relationship between biofilm quantity after incubation with different strains and whether the strains were co-isolate with *S. aureus*. LRT shows the statistic for the likelihood ratio test with the accompanying p – value.

	Df	Deviance	LRT	P -value
EPI	1	60.633	4.039	0.04446*
COR	1	26.734	0.014129	0.9054
CAP	1	10.0080	2.9339	0.08674·

Table 5.2. GLM showing relationship between agr transcription levels after incubation with different strains and whether the strains were co-isolate with *S. aureus*. LRT shows the statistic for the likelihood ratio test with the accompanying p – value.

5.5 Discussion

The majority of nasal *S. epidermidis* isolates were shown to be able to prevent *S. aureus* biofilm formation and inhibit the *S. aureus agr* signal transduction system using the assays described here. However, the ability to inhibit an *in vitro* biofilm did not affect the coexistence of *S. epidermidis* and *S. aureus*. Conversely, *S. epidermidis* strains that could inhibit Agr signaling were significantly more likely to be isolated from *S. aureus* containing communities. Most strains of *S. capitis* could potentially inhibit *S. aureus* Agr signal transduction. Moreover there was a negative association between Agr interfering strains and coexistence with *S. aureus* (Fig. 5.6), although this was not significant ($p = 0.08674$, Table 5.2). Most *S. capitis* strains did not affect biofilm formation and there was no association between strains that did prevent biofilms and *S. aureus* coexistence. No *Corynebacterium* spp. strains were able to prevent biofilm formation or inhibit Agr signal transduction.

Previous studies have shown that biofilm disruption may prevent nasal colonisation by *S. aureus* (Iwase *et al.*, 2010; Quinn *et al.*, 2009). Esp producing strains of *S. epidermidis* were shown to disrupt preexisting biofilms *in vitro* and displace commensal *S. aureus* when they were inoculated into a human nose (Iwase *et al.*, 2010). Furthermore, colonisation of nasal epithelial cells was associated with the ability to form *in vitro* biofilms (Quinn *et al.*, 2009). However, the data from this chapter do not suggest that the potential for biofilm disruption poses a major barrier to nasal colonisation by *S. aureus*. *S. aureus* was just as likely to coexist with strains that inhibited *in vitro* biofilm formation, as those that did not (Table 5.1). These findings are consistent

with histological studies, which showed no evidence of biofilm formation in the *S. aureus* nasal colonisation of cotton rats (Nouwen *et al.*, 2004a; ten Broeke-Smits *et al.*, 2010).

Studies have also shown that interference with Agr signaling in *S. aureus* can affect nasal carriage (Lina *et al.*, 2003). The data in this chapter suggest that strains of *S. epidermidis* that can interfere with *S. aureus agr* transcription were more likely to coexist with *S. aureus* in the nose (Table 5.2). By contrast, strains of *S. capitis* that interfere with *agr* transcription were less likely to coexist with *S. aureus,* although this was not significant ($p = 0.08674$) (Table 5.2). It is interesting that strains of *S. epidermidis* and *S. capitis* that have a similar effect on *agr* transcription, display opposite patterns in coexistence with *S. aureus*. The pattern of *S. capitis/S. aureus* coexistence could be explained by the Agr interference reducing *S. aureus* fitness and thereby preventing coexistence. The pattern observed with *S. epidermidis* whereby Agr interfering strains appear to be significantly more likely to coexist with *S. aureus* is less intuitive. A possible explanation is that Agr interference by *S. epidermidis* is used to counteract a competitive effect that *S. aureus* has in the niche. By this logic, only Agr interfering strains are capable of coexisting with *S. aureus*. Another possibility is that the pattern is simply an artifact of an overarching mechanism driving the distribution of *S. epidermidis* and *S. aureus* e.g. toxin mediated interference competiton (chapter 4).

S. capitis has never previously been reported as an inhibitor of *S. aureus* but the data from this chapter support a theory that it is able to interfere with Agr signaling, and that this interference could coincide with exclusion of *S. aureus* from the niche. A logical explanation for this Agr interference is antagonism of the *S. capitis* AIP with the *S. aureus* Agr system. Agr interference was shown to be dependent on the exact *agr* types of the interacting strains (Lina *et al.*, 2003). *S. aureus* is known to have four *agr* types, *S. epidermidis* has three and *S. capitis* has two, each differing in the amino acid sequence of the post translationally modified AIP (Dufour *et al.*, 2002). Both of the *S. capitis* AIPs share close homology with *S. epidermidis* AIP II (Dufour *et al.*, 2002). Lina *et al.* (2004) showed that *S. epidermidis se*AIP II can inhibit the RNAIII transcription in *S. aureus* strains which possess *S. aureus sa*AIP II. The similarity between both *S. capitis* AIPs and *S. epidermidis* seAIP II suggest that Agr interference between *S. capitis* and *S. aureus* is possible. However, this similarity currently can only account for inhibition between *S. capitis* and one out of four *S. aureus* Agr types. Furthermore, although the sequences of the *S. capitis* AIPs and the *S. epidermidis se*AIP II are similar, there is no empirical evidence for their direct interaction.

The implications of *agr* interference are not only important for survival of *S. aureus,* but also for virulence. Most secreted proteins and virulence factors are produced in late exponential growth and as such are regulated by quorum sensing (Foster, 2009). Disruption of Agr was shown in an animal model to significantly reduce virulence (Fleming *et al.*, 2006). Interestingly,

the data from this chapter suggest that Agr interfering strains of *S. epidermidis* are significantly more likely to coexist with *S. aureus.* Further work is required to elucidate whether coexistence results in reduced *agr* transcription in the niche and indeed, a lower chance of suffering from an *S. aureus* infection. Even if *agr* was downregulated in the niche, it seems unlikely that an infection would be less severe, as the interfering organism would have to also be present at the infection site to reduce virulence (Fleming *et al.*, 2006). However, it seems possible that if Agr was not activated in the niche, there would be less chance of dissemination of *S. aureus* cells and a lower expression of virulence genes which could reduce the chance of an infection developing (Foster, 2005; Foster, 2009).

The data from this study do not suggest any possible mechanism for the exclusion of *S. aureus* from the niche by *Corynebacterium* spp. The fact that this exclusion occurs has been well documented, but as yet, no underlying mechanism has been found is surprising. That exclusion cannot be found when studying *Corynebacterium* spp. in isolation and *in vitro* may suggest an antagonism that is brought about by an intermediary as in apparent competition (Bonsall & Hassell, 1997; Morris *et al.*, 2004). The intermediary could be another species in the community, or the human host. This kind of competition has rarely been described in bacteria, except for an apparent interaction between *Strep. pneumoniae* and *S. aureus* mediated by bacteriophage (Selva *et al.*, 2009). Alternatively, apparent competition could be mediated by the host immune system, whereby pathogens illicit immune responses to target competitors (Brown *et al.*, 2007). Equally, the exclusion

could be brought about by a novel mechanism and RNA transcriptome analyses of co-cultured cells might yield clues to pathways modulated in the presence of corynebacteria.

While inferences can be suggested from this study, the *in vitro* methodologies used mean that *in vivo* interactions cannot be predicted with certainty. Animal models could be used to more effectively investigate Agr expression *in vivo* by using a green fluorescent protein reporter plasmid (Zhao *et al.*, 2001). Biofilms are more difficult to study using *in vivo* models, although they can be grown and measured on human epithelial cells (Moreau-Marquis *et al.*, 2010) which would more closely resemble natural conditions. Furthermore, the methods described here cannot discern whether the observed negative effects on biofilm formation where not caused by toxins in the supernatant causing cell death.

There was a high level of variability between the SH1000 controls used in the three biofilm assays (Fig. 5.1 – 5.3), although the values for the media only control remained relatively constant. SH1000 supernatant was used to control for the effects of nutrient depletion, although biofilm formation could be affected by AIPs which could have been present in the SH1000 supernatant. Differing quantities of AIP in the supernatant may account for the variability, as *agr* upregulation caused by high AIP concentrations does affect biofilm formation and adherence to surfaces (Vuong *et al.*, 2000). The media negative control contained 50% single strength BHI in place of any nutrient depleted supernatant. It may have been more suitable to also use a

control that contained 50% sterile water instead of BHI media, to represent the absolute lower limit of nutrient depletion. In the *agr* transcription assays, four controls were used; media only, SH1000 supernatant, *E. faecalis* supernatant (L058) and *B. cereus* supernatant (L001). *E. faecalis* and *B. cereus* were selected as they have not shown evidence of Agr interference. *B. cereus* does not have a homologous quorum sensing system, instead relying on the competence system for cell density sensing (Kramer *et al.*, 2007). *E. faecalis* utilises pheromones which are structurally distinct from the staphylococcal AIP (Nakayama *et al.*, 2001a), and are hence unlikely to interfere with *S. aureus* signal transduction (Mayville *et al.*, 1999). A media only control was included to categorically control against the effect of any Agr interference. However, a control containing 50% sterile water may have been more appropriate to also control for the absolute lower limits of nutrient depletion. Using such a control would probably have negated the need for the media only control and the supernatants from *E. faecalis* and *B. cereus.* The SH1000 supernatant controlled for the possibility that AIPs in the supernatant could artificially elevate *agr* transcription.

The data from this chapter may support the ideas that Agr interference between *S. epidermidis* and *S. aureus* play a role in nasal colonisation. The data may also suggest an Agr interference model for the exclusion of *S. aureus* from the niche by *S. capitis.* However, the previously discussed limitations relating to the suitability of the controls need to be addressed before more accurate conclusions can be drawn.

Chapter 6: How might inhibitory nasal isolates affect the colonisation dynamics of *S. aureus*?

6.1 Introduction

Ecological theory proposes that interference competition can both promote and prevent invasion of resident communities (Adams *et al.*, 1979; Chao & Levin, 1981; Durrett & Levin, 1994; Duyck *et al.*, 2006; Frank, 1994). The *in vitro* data presented in chapters 4 and 5 support the theory that bacterial isolates from nasal microbial communities utilise interference competition, either by killing competitors (chapter 4) or by interfering with cellular processes to reduce competitor fitness (chapter 5). Here, an experimental approach was taken to investigate the role of interference competition in the *in vitro* ecological interactions of *S. aureus* and *S. epidermidis*. These species were the most abundant species isolated from the anterior nares of 60 volunteers during sampling in this study (chapter 3), being present in 21 (35%) and 59 (98%) of the nasal communities, respectively. Inhibition of *S. aureus* (chapters 4 & 5) by *S. epidermidis* isolates potentially contributes to determining the distribution of *S. aureus*, such that *S. aureus* is less likely to inhabit communities containing inhibitor-producing *S. epidermidis* isolates (chapter 4). This pattern could be driven either by a resident, inhibitor-producing *S. epidermidis* preventing invasion of susceptible *S. aureus,* or by an invading inhibitor-producing *S. epidermidis* displacing a resident susceptible *S. aureus* population. To test these two possible scenarios, reciprocal invasion-from-rare experiments (Chao & Levin, 1981) were carried

out between inhibitor-producing or non-inhibitor-producing nasal isolates of *S. epidermidis* and the susceptible lab strain of *S. aureus* SH1000.

In bacterial communities, interference competition is generally caused by costly environmentally secreted toxins, and therefore is likely to be affected by spatial population structure. Chao and Levin (1981) tested the effect of spatial structure on invasion by bacteriocin producing *E. coli* strains into populations of susceptible *E. coli* strains. These experiments demonstrated that in spatially structured environments (agar plates) bacteriocin producers could invade-from-rare (frequency of 0.001) into bacteriocin-sensitive populations. By contrast, in the absence of spatial structure (shaken liquid broth) much higher initial frequencies of producers (frequency of 0.1) were required to allow successful invasion into a population of susceptibles. In spatially structured environments, spatial clustering favoured bacteriocin producers because, while the effects of toxins were localised, toxins reached higher local concentrations. As such the benefits of costly toxin production could be accrued even by small founding populations (Chao & Levin, 1981; Greig & Travisano, 2004). Whereas, in spatially unstructured environments, rapid diffusion of the bacteriocin away from producing cells required bacteriocin producers to exceed a higher threshold frequency, before the benefits of bacteriocin production could be realised. Similar frequency dependent effects of invasion of toxin producers were demonstrated in the yeast *Saccharomyces cerevisiae* (Greig & Travisano, 2004). These studies suggest that inhibitor-producing *S. epidermidis* strains should be better able

to invade-from-rare than sensitive strains, and should do so from lower frequencies in more highly spatially structured populations.

Ecological theory proposes that interference competition by a resident species should prevent invasion by a susceptible species (Adams & Traniello, 1981; Doyle *et al.*, 2003). Because the inhibitor kills susceptible immigrants, invaders are unable to sustain a viable population; such hostile environmental patches are often termed black-hole sinks in population ecology (Holt & Gaines, 1992). However, there is potential for a susceptible species to evolve resistance to the inhibitor, yet this has rarely been considered in the context of interference competition. Several theoretical models predict that the likelihood of adaptation to a black-hole sink environment increases with the rate of immigration from the source population (Gomulkiewicz *et al.*, 1999; Holt *et al.*, 2003). This occurs because higher immigration rates increase the probability that immigrants carry beneficial mutations that are preadapted to survive the conditions of the black-hole sink (Holt & Gaines, 1992; Perron *et al.*, 2008). Therefore, invading *S. aureus* populations are more likely to contain mutants resistant to *S. epidermidis* toxins when invading from higher initial densities and / or frequencies. However, the spread of these beneficial resistance mutations is likely to be impeded in more highly spatially structured environments. This is because competition of the beneficial mutant can only occur at the edge of a colony, and as the colony grows, a smaller proportion of the mutant population will be competing with the ancestral genotype (Habets *et al.*, 2007). Taken together, these studies suggest that non-inhibitor-producing

residents are more easily invaded; that resistance of the invader to inhibitory toxins is more likely to evolve when invaders are at a high starting frequency; and that resistant mutants are more likely to invade in unstructured environments.

6.2 Aims

The specific aims of this chapter were to test the following predictions: [i] inhibitor-producing strains of *S. epidermidis* should be better able than non-producing strains to invade sensitive *S. aureus* populations; [ii] the success of invasion by inhibitor-producers will be independent of starting frequency in structured environments, but invasion will only be possible above a threshold frequency on an unstructured medium; [iii] inhibitor-producing *S. epidermidis* strains should be better able than non–inhibitor-producing strains to restrict invasion by *S. aureus*; [iv] when at a high initial frequency *S. aureus* should be more likely to evolve resistance to inhibitors produced by *S. epidermidis*.

6.3 Materials and Methods

Species	Strain identification	Reference
S. aureus	SH1000	(Horsburgh *et al.*, 2002)
S. epidermidis	B155 (inhibitor-producing)	Nasal isolate – this study
S. epidermidis	B180 (inhibitor-producing)	Nasal isolate – this study
S. epidermidis	B035 (non-inhibitor-producing)	Nasal isolate – this study
S. epidermidis	B115 (non-inhibitor-producing)	Nasal isolate – this study

Table 6.1. Strains used in this chapter.

Invasion assay

All nasal isolates were cultured on BHI agar plates prior to the beginning of the invasion experiment. Bacteria were cultured for 18 h on 50 mm diameter BHI agar plates when the lawns of *S. aureus* (SH1000) and *S. epidermidis* strains (resident and invader – Table 6.1) were scraped off the agar plates and suspended in 10 ml of PBS (Table 2.1), (containing approximately 5 x 10^8 cfu ml^{-1} for *S. aureus* and *S. epidermidis*, determined with a colony count) by vortexing thoroughly. The cfu ml^{-1} in each tube was equalised by diluting the cell suspensions in PBS and comparing the OD_{600} of each suspension. The two organisms were then mixed together in a final volume of 10 ml PBS (Table 2.1) with the invader at different frequencies (ratios) to the resident (0.1:1, 0.01:1, 0.001:1). For brevity these ratios are referred to in this chapter as frequencies and only the first number in the ratio pair is used to define each frequency. The mixtures were vortexed thoroughly before 50 µl (containing approximately 2.5 x 10^6 cells) was plated out on 25 ml BHI agar and incubated at 37° C. Three replicate communities were established at each starting frequency. The communities were transferred to a new agar plate every day for 7 days. Half of the plates underwent a spatial structured regime whereby the transfers were made by replica plating with velvet to maintain spatial structure. While the other half of plates underwent a mixed regime whereby the spatial structure was destroyed at every 24 h transfer by scraping the entire bacterial lawn off the plate and transferring to 10 ml of sterile PBS before thoroughly vortexing and pipetting 50 µl onto a new plate to complete the transfer. Each set was performed in triplicate and the cell densities transferred in each regime were comparable. Viable counts for

each isolate were calculated every second day. On the structured plates this was achieved by scraping the remainder of the bacterial lawn after the replica plating and serial diluting in PBS. Colony counts were done on BHI plates and colonies were differentiated by colony morphology and colour.

6.3.1 Doubling time determination

An overnight culture of each strain (Table 6.1) was inoculated (1% inoculum) into 200 µl of BHI broth in a 96 well plate. The 96 well plates were incubated at 37°C for 8 h and OD_{600} readings were taken at 20 min intervals. The doubling time (mins) was then calculated using the following formula where T_d is the doubling time; t_1 and t_2 are two consecutive time points throughout the bacterial growth; and d_1 and d_2 are the corresponding OD_{600} readings at t_1 and t_2.

$$T_d = (t_2 - t_1) * \frac{\log(2)}{\log(\frac{d_2}{d_1})}$$

6.4 Results

6.4.1 Invasion of *S. epidermidis* into *S. aureus* resident populations

To test whether inhibitory nasal isolates of *S. epidermidis* could invade-from-rare, three frequencies (0.1, 0.01 & 0.001) of two independent inhibitor-producing and non-inhibitor-producing isolates were invaded into sensitive *S. aureus* SH1000 resident populations (Fig. 6.1). The selected *S. epidermidis* isolates had doubling times that did not differ significantly from those of SH1000 (Table 6.2). Invasions were carried out for seven days under

structured and mixed conditions. To quantify the success of the invasion, I calculated the invader selection rate constant for each invader using relative bacterial frequencies from day zero and day seven (method 2.18) (Fig. 6.2); negative values indicated that invasion was not possible, whereas positive values indicated the invasion was possible. The invasion time-course data were visualised using plots of the natural log of the invader to resident ratio over time (Fig. 6.1); in addition plots of the comparative invader selection rate constants (Fig. 6.2), and interaction plots of the selection rate constants against the main effects from a multivariate ANOVA (Fig. 6.3) are shown.

6.4.2 Structure favours invasion of inhibitor- producing S. epidermidis

Environmental structure promoted *S. epidermidis* invasion (structure, $F = 322.77$, $p < 0.001$) (Fig. 6.1.A, 6.1.B & 6.3.A), and this effect was stronger for *S. epidermidis* inhibitor-producers than for non-inhibitor-producers (structure x inhibition, $F = 14.29$, $p < 0.001$) (Fig. 6.3.I). *S. epidermidis* was never able to successfully invade under mixed conditions (Fig. 6.2 & 6.3.A). However, *S. epidermidis* was more likely to persist at low frequencies and avoid extinction in mixed environments when initiated at a higher starting frequency (frequency x structure, $F= 13.55$, $p <0.001$).

	doubling time (min)	T-Value	P-Value
SH1000	116.45	NA	NA
B180	116.06	-0.074	1.0000
B155	110.11	-1.203	0.5689
B115	120.49	0.767	0.8579
B035	123.34	1.307	0.4970

Table 6.2. Doubling times of strains used in this study. The doubling times in minutes were compared to SH1000 (*S. aureus*) as a control using a Dunnetts test. There is no significant difference between any of the *S. epidermidis* strains tested and the *S. aureus* strain SH1000 used in this study.

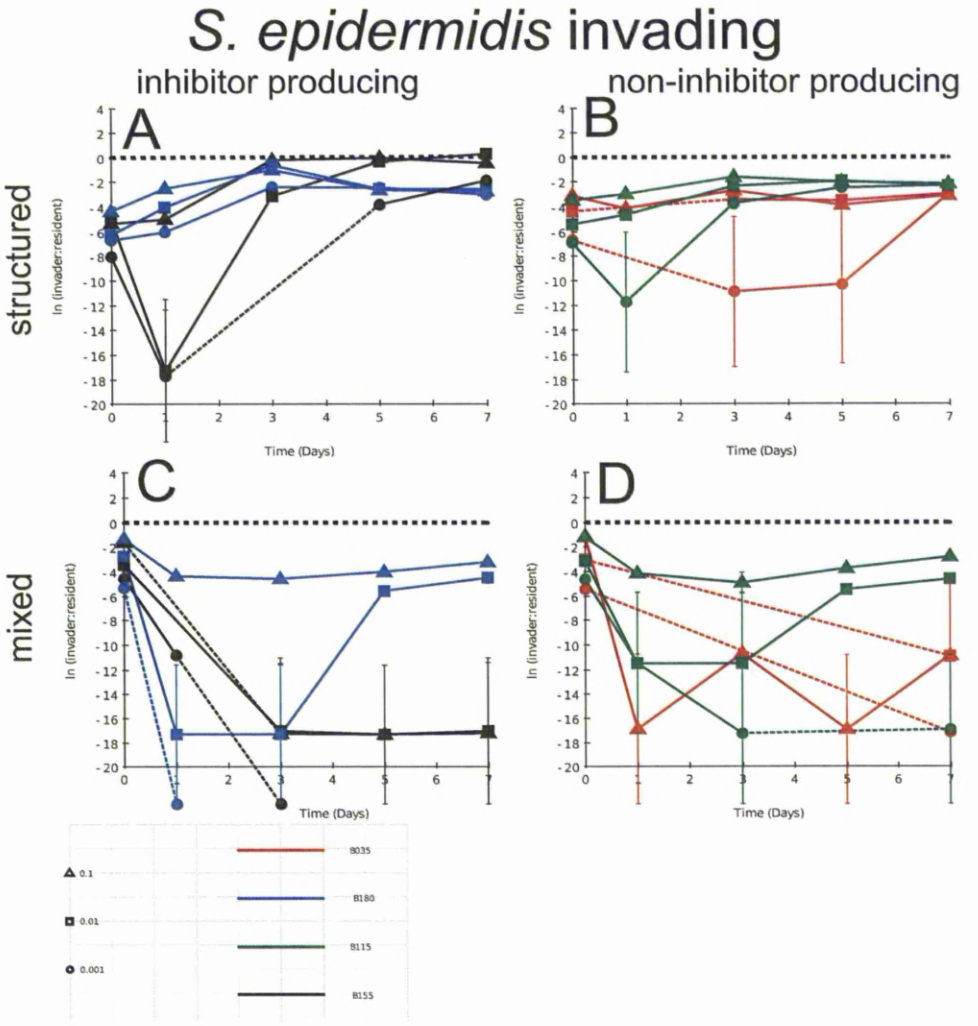


Figure 6.1. Inhibitor-producing (blue and black) and non-inhibitor-producing (red and green) isolates of *S. epidermidis* invading populations of *S. aureus* (SH1000) at frequencies of 0.1 (triangle), 0.01 (square) and 0.001 (circle). Inhibitor-producing *S. epidermidis* isolates (155 & 180) and non-inhibitor-producing *S. epidermidis* isolates (035 & 115) were introduced into a population of *S. aureus* (SH1000) at three different frequencies. This was carried under a spatially structured regime (A and B) and under a mixed regimen (C and D). The x-axis is the time in days and the y-axis is the natural log of the invader to resident ratio. A dotted line in the time course shows when the population dipped below the experiment detection threshold. There is a heavy dotted line at 0 on the y-axis to indicate an equal invader to resident ratio. The line crossing the x-axis symbolises that the population went to extinction.

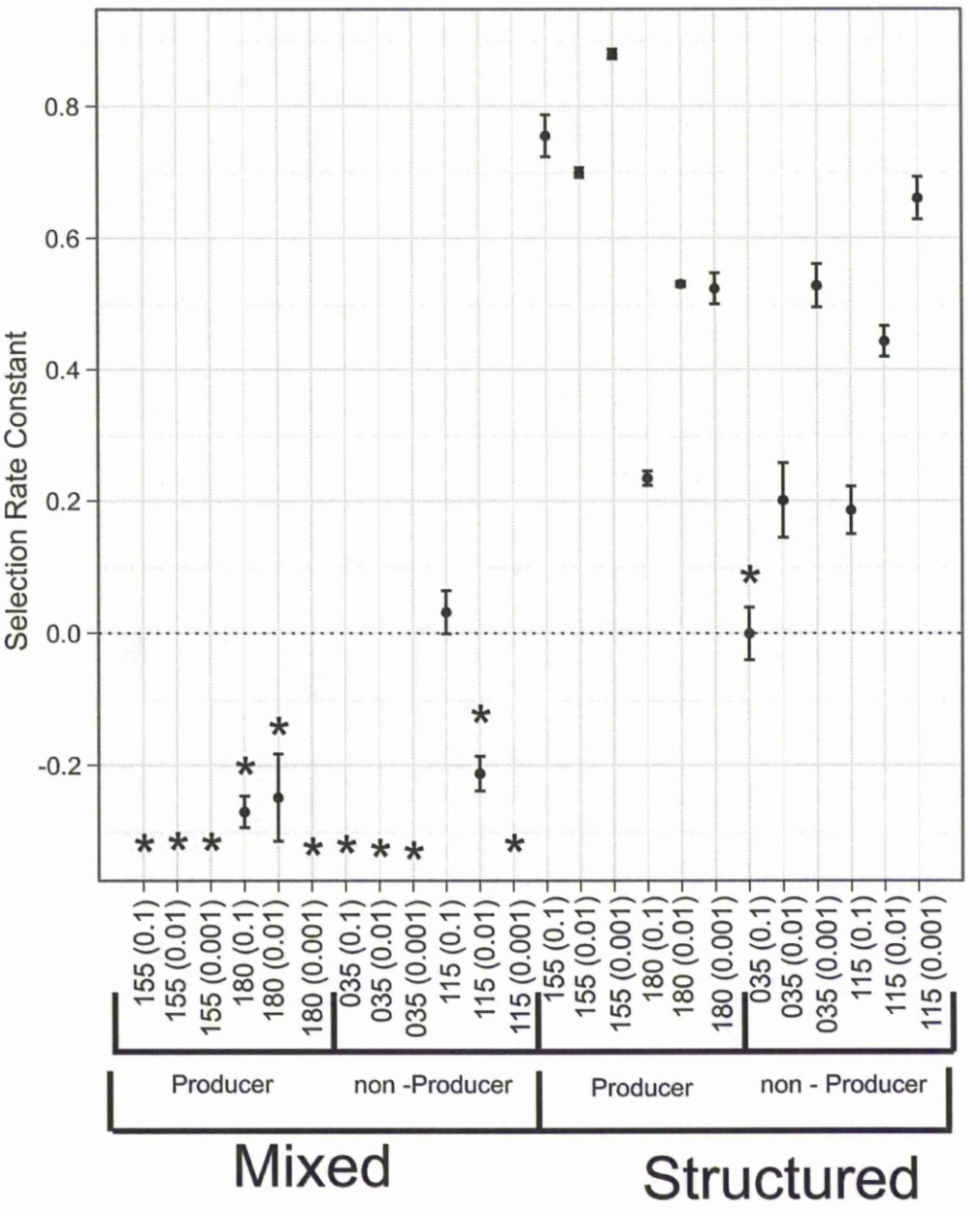


Figure 6.2. Selection rate coefficients for *S. epidermidis* invading populations of *S. aureus* (SH1000). Inhibitor- producing *S. epidermidis* isolates (155 & 180) and non-inhibitor-producing isolates (035 & 115) were invaded into populations of *S. aureus* (SH1000) at relative frequencies of 1 in 10, 1 in 100 and 1 in 1000, denoted by 10, 100, and 1000 respectively on the x – axis label. Each of the invasions was also carried out under a spatially structured treatment and a mixed treatment. Asterisks mark negative selection rate coefficients where invasion did not occur.

	Df	Sum Sq	Mean Sq	F value	P value
frequency	1	0.1382	0.1382	3.4920	0.0662444
structure	1	12.7710	12.7710	322.7662	< 2.2e-16***
inhibition	1	0.1452	0.1452	3.6690	0.0599020
frequency x structure	1	0.5359	0.5359	13.5452	0.0004798***
frequency x inhibition	1	0.0243	0.0243	0.6141	0.4361327
structure x inhibition	1	0.5652	0.5652	14.2852	0.0003474***
frequency x structure x inhibition	1	0.0746	0.0746	1.8858	0.1744717

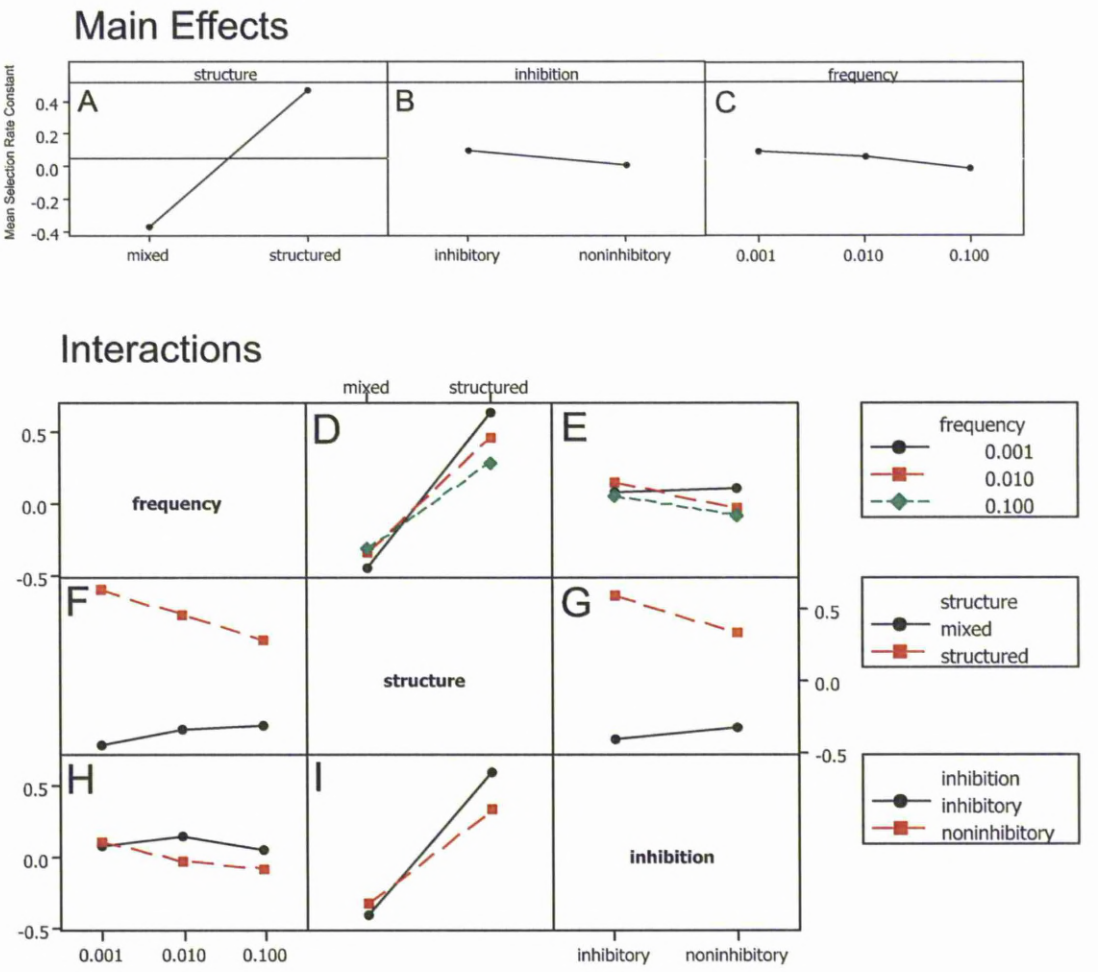


Figure 6.3. Analysis of variance testing the main effects of successful invasion of *S. epidermidis* into populations of *S. aureus*. The Table shows the results of a multifactorial ANOVA. Panels A – B show the model main effects of structure (A), inhibition (B) and frequency (C). Panels D – I show the interactions between the three factors.

6.4.3 Invasion of S. epidermidis is not complete due to evolution of resistance

Under structured conditions, the two invading, inhibitor-producing strains of *S. epidermidis* show different dynamics over time (Fig. 6.1.A). While for all starting frequencies of B155 increase after day 1 and approach a 1:1 invader to resident ratio, B180 (starting frequencies 0.1 and 0.01) increase until day 3, after which they decrease. Spray assays were performed (method 2.6) to test if the fall in frequency of the B180 populations (of starting frequency 0.1 and 0.01) was caused by resistance evolution in the resident *S. aureus* population. Ancestral and evolved resident *S. aureus* clones were sprayed-over ancestral and evolved *S. epidermidis* B180 (Fig. 6.4). These assays show that after seven days, the resident *S. aureus* had evolved resistance to the invading *S. epidermidis* under structured conditions at frequencies of 1 in 10 and 1 in 100 (Fig. 6.4) (Fishers Exact Test $p = 0.0022$). Resistance was not seen in the *S. aureus* resident population when invaded with B180 at a frequency of 1 in 1000 (Fig. 6.4) (Fishers Exact Test $p = 1$). Interestingly, evolved *S. epidermidis* strains (Fig. 6.4.B) produced larger inhibition zones against susceptible *S. aureus* than the ancestral *S. epidermidis* strains (Fig. 6.4.A) (paired T-test $T = 2.69$, $p = 0.03$).

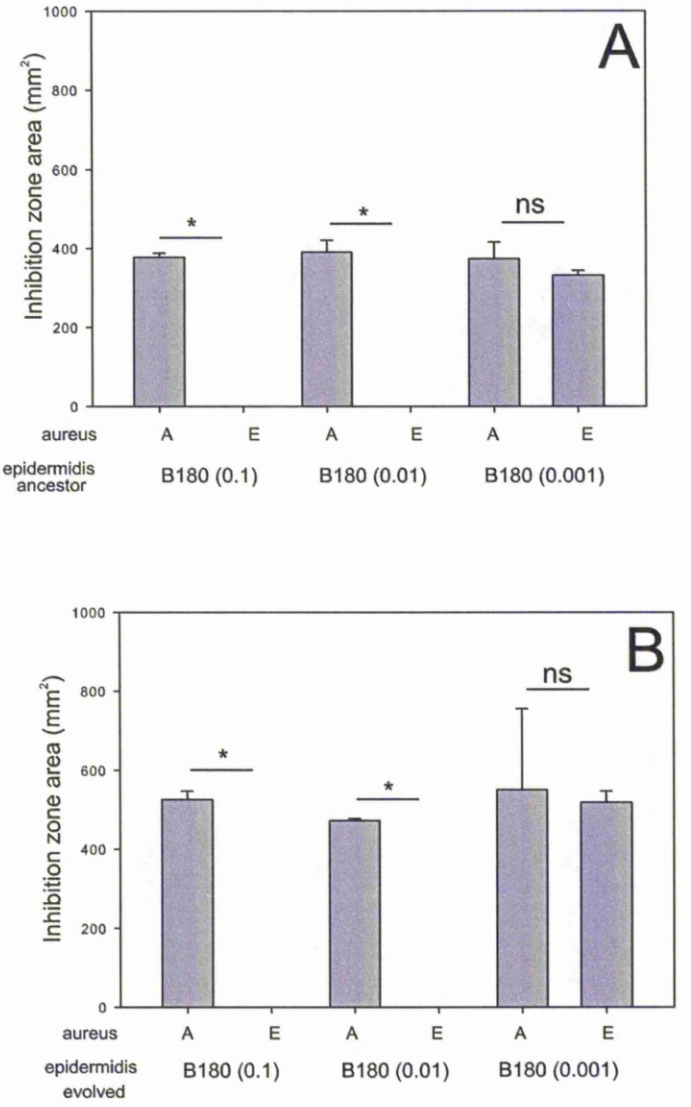


Figure 6.4. Resistance of evolved SH1000 resident after *S. epidermidis* (B180) invasion. Panel A shows inhibition zone produced by the ancestral *S. epidermidis* strains and panel B shows the inhibition zones produced by the evolved *S. epidermidis* strains. Both panel A and B show the inhibition zone area (mm^2) produced by the inhibitor-producing *S. epidermidis* strains against the ancestral SH1000 (A) and the evolved SH1000 (E). Asterisks represent a significant difference between the inhibition zone areas of ancestral (A) and evolved (E) *S. aureus* strains as determined by a Fishers Exact test. Each significance star represents a P value of 0.0022 which is significant when Bonferroni corrected for multiple comparisons with an alpha value of 0.1.

6.4.4 Invasion of *S. aureus* into *S. epidermidis* resident populations

To test if *S. aureus* invasion could be restricted by *S. epidermidis* the reciprocal invasion of *S. aureus* into resident populations of inhibitor-producing and non-inhibitor-producing *S. epidermidis* was also carried out (Fig. 6.5). This tested the hypothesis that inhibitor-producing residents will restrict the invasion of sensitive invaders (Adams & Traniello, 1981; Doyle *et al.*, 2003).

6.4.5 Inhibitor-producing S. epidermidis strains are able to resist invasion, especially in structured environments.

Inhibitor-producing *S. epidermidis* strains are more resistant to invasion than non-inhibitor-producing strains (inhibition, $F = 124.95$, $p < 0.0001$, Fig. 6.7), and restrict invasion more effectively under structured environmental conditions (6.5.A & 6.6) (structure x inhibition, $F = 6.14$, $p < 0.5$, Fig. 6.7). Invasion of *S. aureus* into an inhibitor-producing *S. epidermidis* resident was positively frequency dependent with highest initial frequencies invading the fastest and lower initial frequencies going to extinction (Fig. 6.5.A & 6.5.C) (frequency x inhibition, $F = 46.56$, $p < 0.001$).

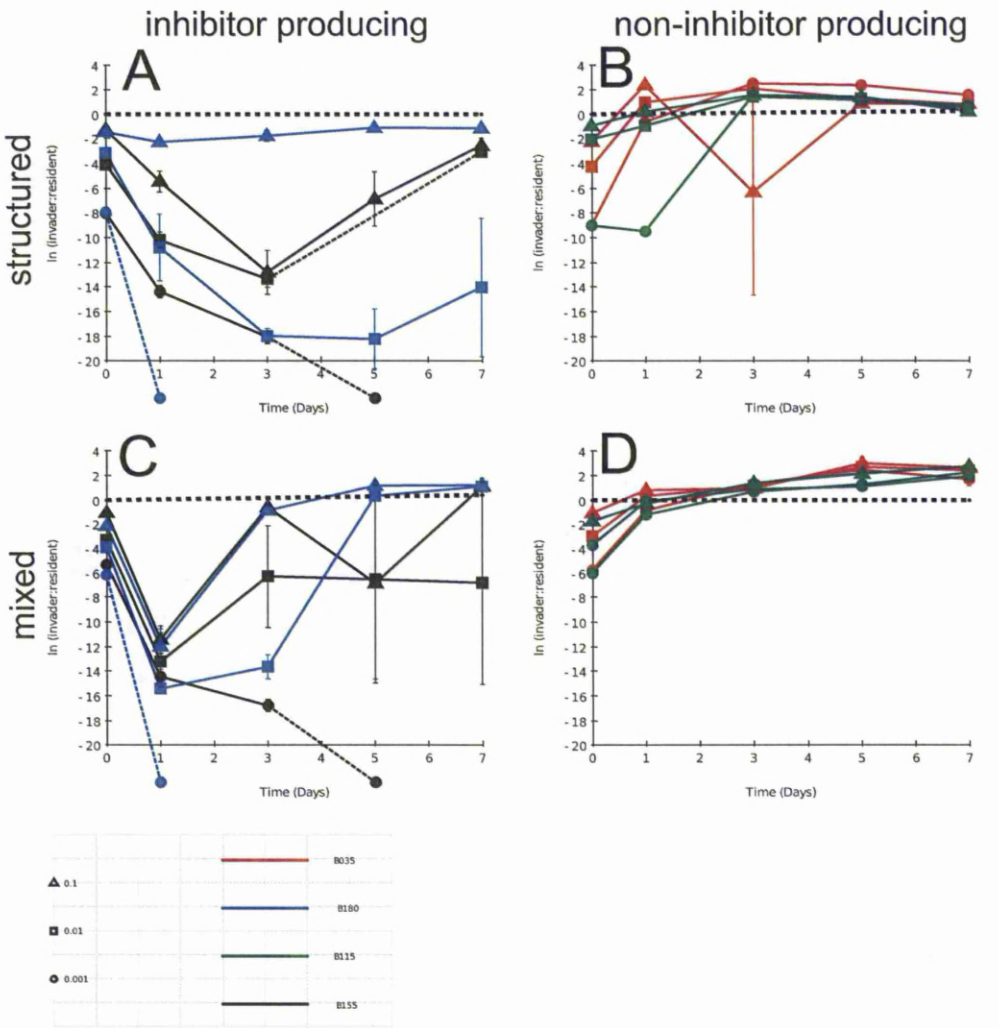


Figure 6.5. *S. aureus* invading populations of inhibitor- producing (blue and black) and non-inhibitor- producing (red and green) *S. epidermidis* at frequencies of 0.1 (triangle), 0.01 (square) and 0.001 (circle). *S. aureus* strain (SH1000) was introduced into two different inhibitor- producing *S. epidermidis* populations (155 & 180), and two different non-inhibitor-producing populations (035 & 115) at three different frequencies. This was carried under a spatially structured regime (A and B) and under a mixed regime (C and D). The x-axis is the time in days and the y-axis is the natural log of the invader to resident ratio. A dotted line in the time course shows when the population dipped below the experiment detection threshold. There is a heavy dotted line at 0 on the y-axis to indicate an equal invader to resident ratio. The line crossing the x-axis symbolises that the population went to extinction.

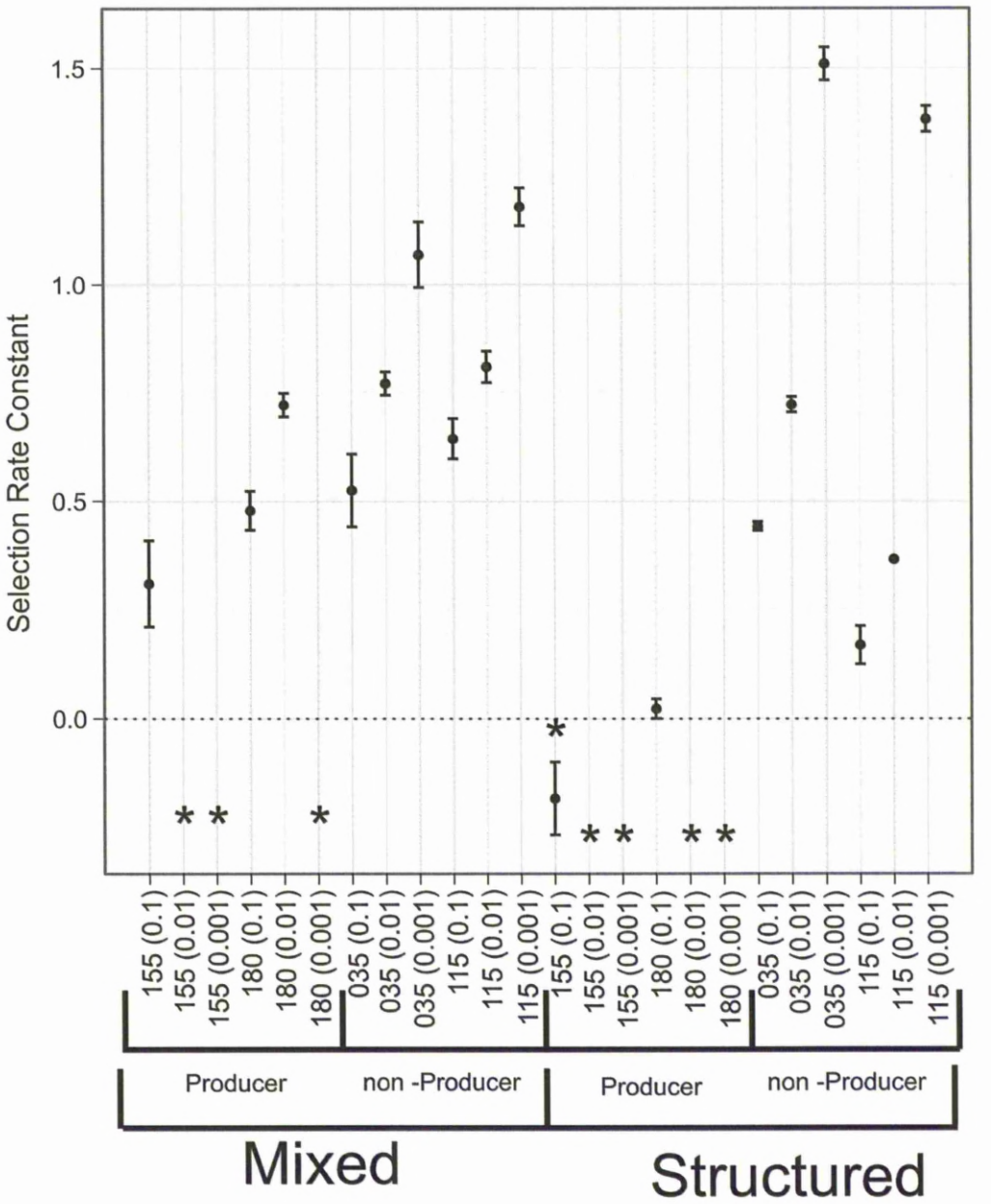


Figure 6.6. Selection rate coefficients for *S. aureus* (SH1000) invading populations of *S. epidermidis*. *S. aureus* was introduced into populations of inhibitor- producing *S. epidermidis* isolates (155 & 180) and non-inhibitor-producing isolates (035 & 115) at relative frequencies of 1 in 10, 1 in 100 and 1 in 1000, denoted by 10, 100, and 1000 respectively on the x – axis label. Each of the invasions was also carried out under a spatially structured treatment and a mixed treatment. Asterisks mark negative selection rate coefficients where invasion did not occur.

	Df	Sum Sq	Mean Sq	F value	P value
frequency	1	2.721	2.721	8.1457	0.005810**
structure	1	2.949	2.949	8.8266	0.004177**
inhibition	1	41.744	41.744	124.9525	< 2.2e-16***
frequency x structure	1	0.007	0.007	0.0198	0.888449
frequency x inhibition	1	15.554	15.554	46.5589	3.794e-09***
structure x inhibition	1	2.051	2.051	6.1382	0.015880*
frequency x structure x inhibition	1	0.398	0.398	1.1900	0.279418

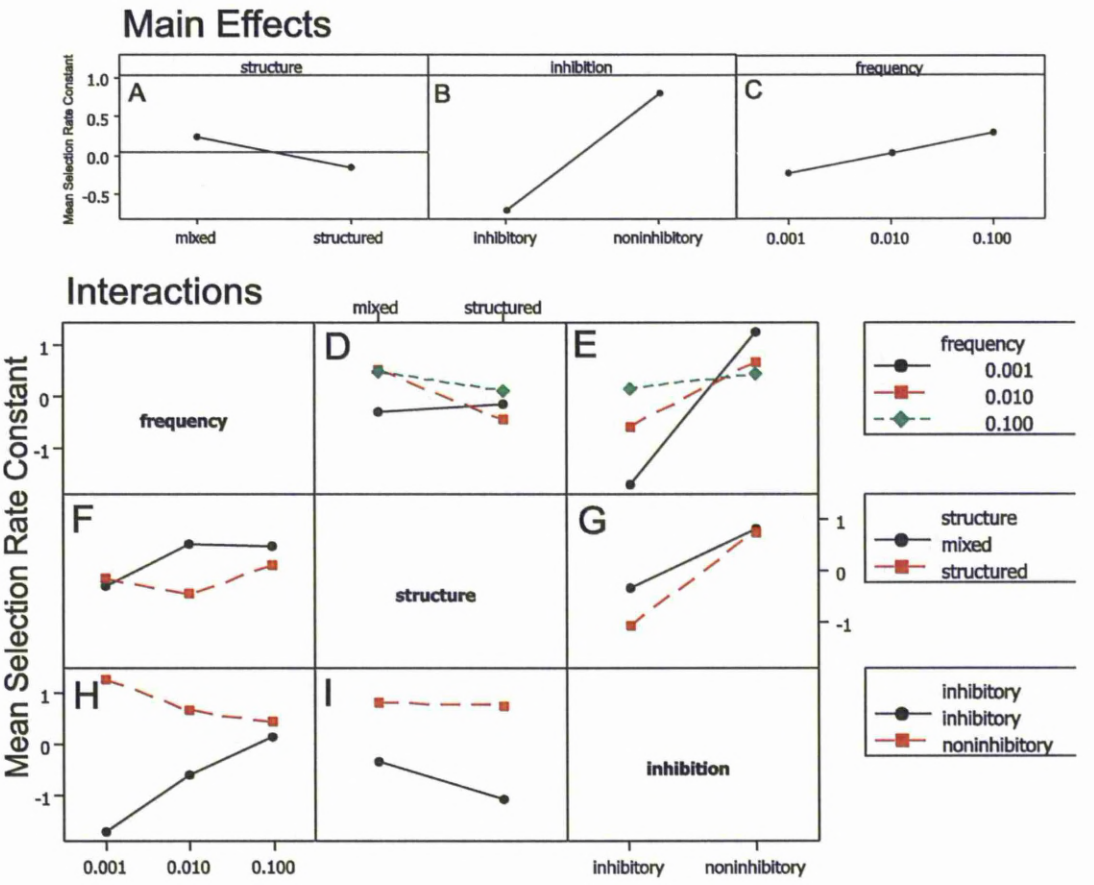
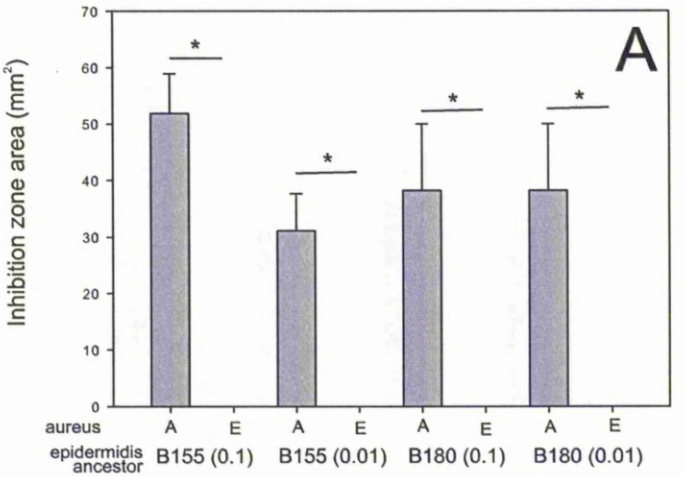


Figure 6.7. Analysis of variance testing the main effects of successful invasion of *S. aureus* into populations of *S. epidermidis*. The Table shows the results of a multifactorial ANOVA. Panels A – B show the model main effects of structure (A), Inhibition (B) and frequency (C). Panels D – I show the interactions between the three factors.

6.4.6 Successful S. aureus invasion is a result of resistance to toxins

As stated above, *S. aureus* was only able to invade inhibitor-producing *S. epidermidis* under mixed conditions (Fig. 6.5.C). To test if the evolution of inhibitory toxin resistance by *S. aureus* was responsible for the invasion in a mixed environment (Fig. 6.6 & 6.5.C) ancestral and evolved *S. arueus* strains were sprayed-over ancestral and evolved *S. epidermidis* inhibitor-producing residents (spray assay, method 2.6). In all cases evolved *S. aureus* were resistant to the *S. epidermidis* inhibitory toxin (Fig. 6.8) (Fishers Exact Test p = 0.0022).



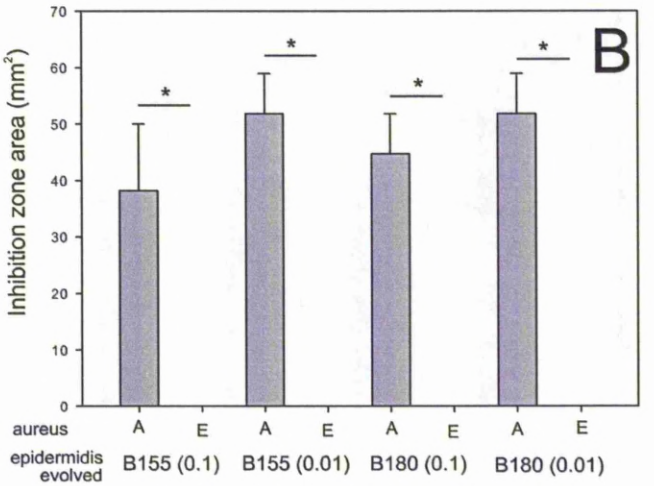


Figure 6.8. Resistance of evolved SH1000 after successful invasion. Panel A shows inhibition zone produced by the ancestral *S. epidermidis* strains and panel B shows the inhibition zones produced by the evolved *S. epidermidis* strains. Both panel A and B show the inhibition zone area (mm^2) produced by the inhibitory *S. epidermidis* strains against the ancestral SH1000 (A) and the evolved SH1000 (E). The * represents a significant difference between the inhibition zone areas of ancestral (A) and evolved (E) *S. aureus* strains as determined by a Fishers Exact test. Each significance star represents a P value of 0.0022 which is significant when Bonferroni corrected for multiple comparisons with an alpha value of 0.1.

6.5 Discussion

These findings demonstrate that inhibitor-producing *S. epidermidis* were able to invade *S. aureus* populations more successfully than non-inhibitor-producing strains, and that this invasion was promoted by environmental structure. However, inhibitor-producing *S. epidermidis* do not completely displace the resident *S. aureus* due to evolution of resistance. Conversely, inhibitor-production by *S. epidermidis* restricted invasion by *S. aureus*, and did so most strongly in structured environments. Where *S. aureus* invasion of inhibitor-producing *S. epidermidis* populations occurred, this resulted from the evolution of inhibitory toxin resistance by *S. aureus*. Invasion of resistant mutants was favoured by high starting frequency and a lack of environmental structure. Taken together these findings suggest that production of inhibitory toxins can both promote invasion by, and prevent invasion into, populations of producers, and that both effects are promoted by environmental structure. Using experimental ecology, I therefore demonstrate a mechanism by which certain members of the resident microbial flora present in the human anterior nares can prevent *S. aureus* from invading nasal communities.

Spatial structure is likely to be an important component of the microbial ecology of the anterior nares. The macrotopography of the nares is very irregular, with many ridges and recesses giving rise to numerous spatially discrete surfaces. The base layer of the nares is comprised of a squamous epithelium upon which microbes colonise and can form spatially discrete groups (Sullivan *et al.* 2003). The data in this study show that this spatial structure potentially plays a crucial role in the community dynamics. If spatial

structure is maintained then inhibitor-producing bacteria can better prevent the invasion of sensitive *S. aureus* providing that the starting frequency of invaders is low (Fig. 6.5.A), which is likely to be the case in invasions of natural communities.

Within a spatially structured environment, the frequency of the invader was also shown to be important. Positive frequency dependence was observed when *S. aureus* was invading inhibitory residents (Fig. 6.1.A & 6.1.C). This probably occurred due to a higher chance of these invading populations containing pre-existing rare resistant mutants. This pattern mirrors a black hole sink environment, where increased immigration rate, which is analogous to increased frequency of the invading population, has been shown to increase the probability of resistance mutants invading a sink habitat (Holt & Gaines, 1992; Perron *et al.*, 2008). Selection is likely to be strong in a black hole sink (Perron *et al.*, 2008) and positive selection for resistant invaders into an inhibitor-producing population is clearly shown at all frequencies (Fig. 6.8). However this was slightly different in the reciprocal experiment when inhibitor-producing *S. epidermidis* were invading resident *S. aureus*. The strength of selection for resistance in the resident population on a structured environment was strongest when the inhibitor-producing invaders were common (Fig. 6.4). This was possibly because more of the resident population was exposed to the invading toxin producers when they were relatively common. Additionally, on a structured medium, the B180 inhibitor-producing invader shows evidence of co-evolution with the *S. aureus* resident, which evolves resistance. The evolved invader shows a stronger

inhibitory activity than the ancestor (Fig. 6.4). It is likely that the inhibitory strain simply upregulates production of the inhibitory toxin, or alternatively this effect could be due to the production of alternative toxins. However, in the absence of knowledge of the mechanism of inhibition, this remains unclear.

Spatial structure also promoted the invasion of non-inhibitor producing strains (Fig. 6.1.B & 6.1.D). The non-inhibitor producing strains used were isolated from the environment and are not genetically identical to the inhibitor-producing strains with the toxin genes knocked out. Therefore it is possible that the non-inhibitor producing strains posses other mechanisms promote their invasion.

It is also difficult to discern the mechanism of *S. aureus'* resistance to the inhibitor-producing strains. Most likely is that the inhibitors produced are cationic antimicrobial peptides e.g. bacteriocins which are prevalent in staphylococci and in particular *S. epidermidis* (Hechard & Sahl, 2002; Schnell *et al.*, 1988; Tagg *et al.*, 1976). In *S. aureus,* resistance to a wide range of cationic antimicrobial peptides are attributed to mutations in the *dlt* (Peshcel *et al.* 1999) and *MprF* (Staubitz *et al.* 2004) genes, which alter the surface charge on the bacterial cell to repel cationic molecules. To investigate this further the *S. epidermidis* toxins would have to be identified using HPLC. Then mutational studies could be carried out using SH1000 *dlt* and *MprF* null mutants to test the hypothesis that these strains would not be sensitive to the toxins produced by *S. epidermidis* and would be able to

invade from rare, even on a structured medium (Peschel *et al.*, 1999; Staubitz *et al.*, 2004).

An additional factor to consider is that within the inhibitor-producing colonies, species are potentially inhibiting their own growth (Pacala, 1986), and therefore the fitness of the invading strain can be reduced over time (Habets *et al.*, 2007). Previous studies have shown that fitness decreases as the frequency of the inhibitor-producing invader increases because the rate of intraspecific competition increases (Burke & Grime, 1996; Crawley, 1990). The data from this chapter show that the ratio of invader to resident can decrease following an initial increase that could indicate a reduction in fitness (Fig. 6.1.A – B180). This decrease in ratio can partly be explained by evolution of resistance (Fig. 6.4) but there is no evidence to suggest that the inhibitor-producing colonies do not also inhibit their own growth as they increase in frequency.

It is important to consider the relevance of an *in vitro* model to the natural environment. This study employed a mixing regime that involved growing the populations on a spatially structured substrate, but regularly destroying the structure during the transfer protocol (Habets *et al.*, 2006). This is arguably more relevant to the nasal environment than the mass habitat used by Chao and Levin (1981), which is essentially a non-viscous liquid culture (Chao & Levin, 1981). However, both studies demonstrate that unstructured environments generally do not favour the production of inhibitory toxins. This displays an interesting view of the dynamics of the microbial community in

the anterior nares, since the spatial structure of the nose is likely to be in a state of unpredictable flux. Structure is expected to be present, but the structure might be randomly broken by changes in water content on the surface of the squamous epithelium. Moreover, changes in the flow of air, mucus and blood through the nasal passages and mechanical disruptions by nose picking may also disrupt spatial structure. Factors that reduce the spatial structuring in nasal communities could weaken the ability of inhibitory resident species to prevent invasion by *S. aureus*.

Chapter 7: Final Discussion

S. aureus is an important opportunistic pathogen carried by approximately 50% of humans (van Belkum *et al.*, 2009). *S. aureus* is not the only species inhabiting the human anterior nares. On the contrary, the nares is colonized with a community of microorganisms in which *S. aureus* must survive for successful carriage. Although many different species are known to reside in the nose, the diversity is very limited when compared with the microbial communities of the oral cavity (Ahn *et al.*, 2011) and gut (Kinross *et al.*, 2011). A major factor in the selection of organisms capable of residing in the nose is proposed to be the high salt content of nasal secretions (Uehara *et al.*, 2000), in which most Gram-negative bacteria survive poorly (Cole *et al.*, 1999). This factor combined with the limited supply of carbon sources (primarily mucin (Cole *et al.*, 2001)) and the high levels of innate immunity factors such as lysozyme, lactoferrin, imunoglobulins (IgA, IgG and IgM) (Cole *et al.*, 1999) and antimicrobial peptides (Peschel & Sahl, 2006) make the nasal environment relatively inhospitable. While interactions between *S. aureus* and several species from the nasal community have been shown experimentally to affect carriage, (Iwase *et al.*, 2010; Lina *et al.*, 2003; Uehara *et al.*, 2000), this thesis highlights potentially important roles of a broad range of species interactions in driving *S. aureus* distribution.

7.1 Community Structure and Species Interactions

Community ecology techniques were used to study interspecies interactions that determine the distribution and abundance of species. Recently two culture-independent surveys of the microbial communities in the anterior nares were carried out, showing the distribution and abundance of the nasal microbial community members (Frank *et al.*, 2010; Wos-Oxley *et al.*, 2010). Furthermore, these studies inferred interactions between *S. aureus* and other species from presence/absence associations. Using similar statistical methods, it was possible to infer species interactions in the metacommunity sampled here. Specifically, significant negative associations with *S. aureus* were observed for the following taxa: *S. capitis, C. propinquum, C. macginleyi, Enterobacter aerogenes, S. epidermidis, Micrococcus* sp., *Bacillus* sp., *C. accolens, S. schleiferi* and *Gemella haemolysans.*

However, the culture-based methods used in this thesis enabled a study beyond simple associations and investigation of the underlying mechanisms dictating species interactions. Several novel interspecific interactions that could account for the distribution of *S. aureus* in the niche were identified between *S. aureus* and the following taxa; *S. epidermidis, S. capitis, C. propinquum, C. accolens* and *Micrococcus* sp.. Negative associations between only the inhibitor-producing subsets of *Micrococcus luteus* and *S. hominis* were uncovered, which was likely to be toxin-mediated (e.g. antibiotic, bacteriocin, lantibiotic) given the nature of the inhibition spray assay used, where no contact between colonies was required for inhibition (chapter 4). Inhibitor-producing strains of *Micrococcus luteus* and *S. hominis*

were significantly less likely to co-exist with *S. aureus* than non-inhibitor-producing isolates of these species (chapter 4). The observed negative association between *S. capitis* and *S. aureus* (chapter 3) was potentially also mediated by interference with the *S. aureus* quorum-sensing system because *S. capitis* strains that did not co-exist with *S. aureus* were more likely to reduce Agr signaling (chapter 5). Since strains of the same species are often highly phenotypically variable, these findings demonstrate clear limitations to using simple species-level presence/absence associations to infer interactions. The overlaying of phenotypic information onto the species distributions provided higher fidelity information over-and-above species-level identifications that has allowed the detection of previously unknown species interactions.

7.2 Toxins, space and invasion

Experimental ecology approaches using cultivable nasal isolates allowed detailed investigation of the ecological factors affecting competition and invasion (Chapter 6). Data from the invasion experiments demonstrates that inhibitor-producing nasal isolates of *S. epidermidis* were better able invade *S. aureus* populations than non-inhibitor-producing nasal isolates, and invade more effectively in a spatially-structured environment. Furthermore, the same inhibitor-producing strains of *S. epidermidis* were able to significantly restrict the invasion of *S. aureus,* especially on a structured medium. Co-evolution was also observed when inhibitor-producing invaders produced stronger inhibition, seemingly in response to the evolution of resistance in the resident population. While a surrogate lab strain of *S aureus* was used, the relevance

of the findings are potentially supported by the fact that nasal communities harbouring many inhibitor-producing isolates (inhibitory to SH1000, the same lab strain), were less likely to contain *S. aureus* (chapter 4). These findings need to be confirmed with several nasal *S. aureus* isolates in the future. Interestingly, invasion by *S. aureus* into inhibitor-producing populations of *S. epidermidis* was observed, and was possible due to the evolution of inhibitor-resistance. The evolution of inhibitor-resistance was positive frequency-dependent, reflecting that inhibitor-resistance mutations were likely to have been pre-existing at low frequency in the inoculum. Furthermore, spatial structure restricted the invasion of these beneficial mutations. Taken together with the finding that inhibitor-producing *S. epidermidis* strains from the end of the experiment produced larger zones of clearing, this suggests the potential for coevolution between the inhibitor-producing species and its victim.

7.3 Limitations

7.3.1 Culture versus Culture-independent Based Methods

Culture based methods have well documented limitations in their use to explore microbial communities (Frank *et al.*, 2010; Wos-Oxley *et al.*, 2010). Indeed, this study has highlighted those limitations with the failure to isolate propionibacteria from any of the communities sampled, which is likely a result of the culturing strategy. This strongly suggests that, although culture dependent methods have many benefits, the study of microbial communities is greatly enhanced by culture-independent approaches.

Advances in sequencing technologies are making the molecular elucidation of interactions more of a possibility. One example of this is how the genotypes present in a community can help predict some of the interactions that might occur. Furthermore, using RNA sequencing, detection of the levels of transcription of an organism is possible (van Vliet, 2010). Currently this technology can only be used to determine the level of transcription of an entire community or an entire metacommunity. This means that it is difficult to pick apart individual interactions. However, it seems like this will be possible in the future, with the ability to isolate and analyse the transcriptome of single bacterial cells from a bacterial population (Güell *et al.*, 2011; Kang *et al.*, 2011). Also, as sequencing technology progresses, knowing the genome sequences of the species present will help to understand the potential interactions that may occur in the niche. If the genes are known and their mode of action is understood, then it is possible to predict potential interactions between species. However, using *in vivo*, culture based techniques is essential for confirming genomic-based predictions. Furthermore, the making of these predictions rely heavily on *in vitro* studies having previously identified the mode of action of the genes. Development of more high throughput and realistic techniques are required for the more in depth study of microbial communities in a more natural environment. These developments can inform culture-independent studies. An example of this is in the human gut, where models have been constructed to simulate, as closely as possible the conditions in the human stomach and colon which have allowed the study of the effect of drugs on the microbial flora of the gut (Baines *et al.*, 2009; Baines *et al.*, 2011).

Though interactions *in vitro* are relatively easy to test for and can be good indicators of interactions *in vivo* (Iwase *et al.*, 2010), there is no way to accurately predict how and when interactions occur *in vivo*, and how these interactions affect the bacterial communities in the nose. An example of this is that some antibiotics may simply be signaling molecules in their natural environment, and never reach inhibitor concentrations (Linares *et al.*, 2006). However, *in vitro* experiments of antibiotics almost always suggest that they are inhibitory (Rasool & Wimpenny, 1982; Turpin *et al.*, 1992; Wiener, 2000). This suggests that apparent antagonistic interactions *in vitro* are not necessarily antagonistic in the niche. However, *in vitro* models provide much greater flexibility in the experiments that can be done, and are essential precursor to more realistic models using animals and human volunteers.

7.3.2 Use of the S. aureus Lab Strain – SH1000

Since bacteria can transfer genetic elements so readily (de la Cruz & Davies, 2000; Ochman *et al.*, 2000; Thomas & Nielsen, 2005) it seems clear that there is no strain that will be representative of the whole species. While a benefit of this study was that naturally occurring strains were examined, a surrogate lab strain of *S. aureus* was used in place of a natural isolate for consistency in inhibition screen (chapter 4). This also made the screening of a large number of natural nasal isolates possible. Ideally, a surrogate *S. aureus* strain should be representative of all the *S. aureus* strains isolated in the study and genetically well defined. The indicator strain used in this study (SH1000) is unlikely to be representative of the species isolated, given the

high genotypic diversity of natural *S. aureus* isolates (Moore & Lindsay, 2001; Peacock *et al.*, 2000; Sakwinska *et al.*, 2009). Future work needs to be undertaken to test if *S. aureus* nasal isolates show the same patterns as SH1000. However SH1000 is genetically well-defined (Horsburgh *et al.*, 2002). This creates an opportunity to do future studies on the gene regulation of *S. aureus* in response to challenge from inhibitory strains, and to genetically define the resistance mechanism.

7.4 Wider implications

7.4.1 Diagnostic Uses and Infection Prevention

Microbial ecology could be used to assess the risk of a hospital patient acquiring a *S. aureus* infection, or becoming a nasal carrier. Culture dependent techniques may be used to understand the dynamics of the microbial community to the extent where knowing the community composition in advance may be able to predict colonisation probabilities. Culture independent techniques could be used to rapidly identify the members of the microbial flora, and important factors such as bacteriocin production. This could open up a whole new avenue of treatment options, and make personalized medicine a reality (Rizkallah *et al.*, 2010).

7.4.2 Treatment and Control of S. aureus Carriage

The use of ecology to study pathogens in the niche has potential to impact public health, by developing novel strategies to reduce carriage of pathogens. Biological control has been extensively studied in agriculture and the environment (Birdsall & Markin, 2010; Joyce *et al.*, 2010; Rosa-Magri *et*

al., 2010; Waheed & Khilare, 2010). Pro and prebiotics have been studied in the human gut due to the health benefits associated with particular microbial flora, and problems associated with others (Stoidis *et al.*, 2011; Zihler *et al.*, 2010). While the use of probiotics have not been successful in significantly altering the microbial consortia in the human gut, prebiotics have been shown to have much stronger effects (Collins & Gibson, 1999). Phage therapy has been used in the prevention of infections (Capparelli *et al.*, 2007; Tothova *et al.*, 2011) but the use of biological control to change the microbial flora of the human nose has not been considered. The use of microbial ecology could be used to discover novel prebiotics, to promote the nasal carriage of organisms that actively inhibit pathogens such as *S. aureus*. Studies have shown that organisms such as corynebacteria and *S. epidermidis* are present in the anterior nares of humans at the exclusion of *S. aureus* (Frank *et al.*, 2010; Iwase *et al.*, 2010; Lina *et al.*, 2003; Uehara *et al.*, 2000). Elucidating the exact mechanism of these interactions and promoting the existence of species that cause these negative associations could prove to be a novel therapy in the future. In practice however, intentionally changing the microbial flora of the human nose could have limitations. Firstly, as identified by this study and the recent studies by Frank (Frank *et al.*, 2010) and Wos-Oxley (Wos-Oxley *et al.*, 2010), beta diversity among the microbial communities is high, making it difficult to produce a general pro or prebiotic suitable for every nasal community. Furthermore, even if short-term changes in the microbial communities could be predicted, the long term changes and the associated health impact would be uncertain. Another important factor is that *S. epidermidis,* one of the species identified as having a negative effect

S. aureus nasal carriage (chapter 3, 4 & 6) (Frank *et al.*, 2010; Iwase *et al.*, 2010; Lina *et al.*, 2003) is an opportunistic pathogen (Curtin & Donlan, 2006; Otto, 2009; Rogers *et al.*, 2009). Promoting the growth of *S. epidermidis* may prevent infections caused by *S. aureus* by preventing carriage but in turn it has the potential to cause infections of its own.

By using drugs that target community members affecting *S. aureus*, the ability of *S. aureus* to survive in that community can also be affected. Many studies have looked at the interactions between microogranisms and their potential implications in the niche (Iwase *et al.*, 2010; Margolis, 2009; Selva *et al.*, 2009; Uehara *et al.*, 2000). However, very few studies use the same isolates from a metacommunity survey to identify interactions *in vitro*. In this study, the interactions found are from organisms that were originally isolated from the niche which has shown to be important in discovering new interactions.

Chapter 8: Appendix 1

Nose ID	Species	ID Method	Density (cfu / nose)
270	*S. epidermidis*	16S	$2.20X10^{4}$
	S. aureus	API Staph	$2.00X10^{2}$
259	*S. epidermidis*	API Staph	$1.86X10^{3}$
	S. aureus	API Staph	$8.40X10^{2}$
294	*S. epidermidis*	API Staph	$1.02X10^{4}$
	S. aureus	API Staph	$5.60X10^{3}$
	S. equorum	16S	$9.00X10^{2}$
264	*S. epidermidis*	16S	$9.10X10^{4}$
	S. aureus	API Staph	$2.41X10^{4}$
266	*S. epidermidis*	API Staph	$6.40X10^{3}$
	S. aureus	API Staph	$2.40X10^{3}$
284	*S. epidermidis*	16S	$1.02X10^{3}$
	S. aureus	16S	$3.40X10^{2}$
	Corynebacterium coyleae	16S	$2.80X10^{2}$
327	*S. aureus*	API Staph	$5.30X10^{2}$
286	*S. aureus*	API Staph	$9.60X10^{3}$
	S. saprophyticus	API Staph	$8.00X10^{1}$
	Kocuria kristinae	API Staph	$4.90X10^{3}$
334	*S. epidermidis*	16S	$1.20X10^{4}$
	S. aureus	API Staph	$1.44X10^{5}$
	S. lugdunensis	API Staph	$7.00X10^{4}$
293	*S. epidermidis*	API Staph	$1.20X10^{4}$
	S. hominis	API Staph	$5.00X10^{1}$
281	*S. epidermidis*	API Staph	$1.21X10^{4}$
	S. hominis	API Staph	$3.00X10^{2}$
340	*S. epidermidis*	16S	$1.60X10^{2}$
	S. hominis	API Staph	$2.00X10^{1}$
	Enterococcus faecalis	16S	$2.65X10^{3}$
301	*S. epidermidis*	API Staph	$7.20X10^{2}$
	S. hominis	16S	$2.00X10^{1}$
	S. simulans	API Staph	$4.00X10^{1}$
263	*S. epidermidis*	API Staph	$1.30X10^{3}$
272	*S. epidermidis*	16S	$7.70X10^{2}$
	Citrobacter koseri	16S	$2.00X10^{1}$
276	*S. epidermidis*	16S	$4.00X10^{1}$
256	*S. epidermidis*	API Staph	$9.60X10^{2}$
287	*S. epidermidis*	API Staph	$6.40X10^{4}$
	S. hominis	API Staph	$6.30X10^{4}$
	S. aureus	16S	$6.20X10^{3}$
299	*S. epidermidis*	API Staph	$3.90X10^{3}$
	S. hominis	API Staph	$2.70X10^{2}$
	S. aureus	API Staph	$2.00X10^{1}$
	Citrobacter freundii	16S	$2.00X10^{1}$
258	*S. epidermidis*	16S	$7.30X10^{4}$
	S. aureus	API Staph	$4.30X10^{3}$
	S. capitis	API Staph	$9.00X10^{2}$
337	*S. epidermidis*	API Staph	$2.26X10^{3}$
	S. capitis	API Staph	$5.00X10^{1}$
274	*S. epidermidis*	API Staph	$5.10X10^{4}$
	S. aureus	16S	$7.60X10^{5}$
	S. pasteuri	16S	$1.10X10^{2}$
297	*S. epidermidis*	16S	$5.60X10^{2}$
	Moraxella nonliquefaciens	16S	$6.00X10^{1}$

	S. pasteuri	16S	$2.00X10^{1}$
315	*S. epidermidis*	16S	$1.20X10^{4}$
	S. aureus	16S	$3.40X10^{2}$
	S. warneri	API Staph	$2.00X10^{1}$
255	*S. epidermidis*	16S	$4.00X10^{3}$
	S. aureus	API Staph	$6.70X10^{3}$
	S. warneri	API Staph	$3.20X10^{2}$
291	*S. epidermidis*	16S	$7.80X10^{4}$
	Micrococcus sp.	API Staph	$7.40X10^{4}$
	S. warneri	API Staph	$4.00X10^{3}$
	S. aureus	API Staph	$3.30X10^{4}$
278	*S. epidermidis*	API Staph	$1.76X10^{4}$
	S. aureus	16S	$6.90X10^{3}$
	S. warneri	API Staph	$1.00X10^{2}$
	Corynebacterium smegmentosum	16S	$7.90X10^{3}$
324	*S. epidermidis*	API Staph	$6.30X10^{2}$
	S. aureus	API Staph	$2.90X10^{2}$
	Streptococcus sp.	16S	$6.90X10^{2}$
	S. lugdunensis	API Staph	$2.00X10^{1}$
298	*S. epidermidis*	API Staph	$7.80X10^{4}$
	S. aureus	API Staph	$8.40X10^{4}$
	Streptococcus sp	API Staph	$1.79X10^{4}$
262	*S. epidermidis*	API Staph	$5.13X10^{3}$
	M. luteus	16S	$1.00X10^{2}$
	Corynebacterium macguinleyi	16S	$1.03X10^{3}$
	S. lugdunensis	API Staph	$1.75X10^{3}$
268	*S. epidermidis*	API Staph	$9.80X10^{2}$
	Micrococcus spp.	API Staph	$2.00X10^{1}$
	S. lugdunensis	16S	$1.42X10^{3}$
295	*S. epidermidis*	API Staph	$1.96X10^{3}$
	Corynebacterium accolens	16S	$6.70X10^{2}$
267	*S. epidermidis*	API Staph	$5.80X10^{2}$
	Corynebacterium accolens	16S	$1.09X10^{4}$
	S. sciuri	API Staph	$1.00X10^{2}$
280	*S. epidermidis*	API Staph	$2.84X10^{4}$
	Corynebacterium pseudodiphthericum	16S	$8.70X10^{3}$
273	*S. epidermidis*	API Staph	$2.22X10^{5}$
	Corynebacterium propinquum	16S	$1.11X10^{6}$
	Streptococcus sp.	API Staph	$7.60X10^{4}$
	S. lentus	API Staph	$4.00X10^{1}$
	S. haemolyticus	API Staph	$4.00X10^{3}$
289	*S. epidermidis*	16S	$1.01X10^{4}$
	Corynebacterium sp.	16S	$6.00X10^{2}$
	Streptococcus pyogenes	16S	$6.50X10^{3}$
257	*S. epidermidis*	API Staph	$4.00X10^{2}$
	Corynebacterium sp.	16S	$2.40X10^{2}$
	Bacillus spp.	16S	$2.00X10^{1}$
	Streptococcus sp.	16S	$2.80X10^{2}$
313	*S. epidermidis*	16S	$1.40X10^{2}$
	S. capitis	API Staph	$2.00X10^{2}$
	Streptococcus mitis	16S	$1.40X10^{2}$
	Kocuria cristinae	16S	$2.00X10^{1}$
	Brevidobacterium sp	16S	$2.00X10^{1}$
319	*S. epidermidis*	API Staph	$8.10X10^{2}$
	Streptococcus sp.	16S	$3.60X10^{2}$
	Enterobacter aerogenes	16S	$5.90X10^{2}$

269	*S. epidermidis*	API Staph	$6.30X10^{3}$
	S. capitis	API Staph	$2.00X10^{1}$
	Corynebacterium sp.	16S	$9.10X10^{3}$
	S. pasteuri	16S	$1.40X10^{2}$
250	*S. epidermidis*	16S	$4.20X10^{4}$
	Micrococcus sp.	API Staph	$1.40X10^{2}$
	S. capitis	API Staph	$9.00X10^{2}$
	Corynebacterium smegmentosum	16S	$3.20X10^{4}$
265	*S. epidermidis*	API Staph	$1.70X10^{3}$
	S. capitis	API Staph	$4.00X10^{2}$
	S. warneri	API Staph	$4.70X10^{3}$
	Corynebacterium accolens	16S	$6.10X10^{4}$
275	*S. epidermidis*	16S	$1.22X10^{4}$
	S. warneri	API Staph	$1.10X10^{2}$
	Corynebacterium propinquum	16S	$3.60X10^{3}$
	S. schleiferi	API Staph	$3.00X10^{2}$
283	*S. epidermidis*	16S	$7.00X10^{5}$
	Micrococcus sp.	API Staph	$3.94X10^{6}$
	Corynebacterium accolens	16S	$5.55X10^{6}$
	S. aureus	API Staph	$5.00X10^{2}$
285	*S. epidermidis*	API Staph	$1.25X10^{3}$
	Micrococcus luteus	16S	$2.00X10^{1}$
	S. aureus	16S	$1.20X10^{2}$
	Corynebacterium sp.	16S	$6.64X10^{3}$
292	*S. epidermidis*	API Staph	$7.70X10^{2}$
	S. aureus	API Staph	$2.00X10^{1}$
	M. luteus	API Staph	$3.00X10^{2}$
288	*S. epidermidis*	API Staph	$1.30X10^{2}$
	Micrococcus luteus	16S	$2.00X10^{1}$
	S. aureus	API Staph	$2.00X10^{1}$
	Bacillus simplex	16S	$2.00X10^{1}$
	Corynebacterium accolens	16S	$6.00X10^{1}$
296	*S. epidermidis*	API Staph	$1.30X10^{5}$
	Micrococcus sp.	API Staph	$3.00X10^{1}$
	Corynebacterium accolens	16S	$2.40X10^{4}$
	Bacillus sp	16S	$6.00X10^{5}$
254	*S. epidermidis*	API Staph	$1.97X10^{4}$
	Micrococcus luteus	API Staph	$4.00X10^{1}$
	Bacillus sp.	16S	$1.60X10^{2}$
	Corynebacterium macginleyi	16S	$6.80X10^{4}$
253	*S. epidermidis*	API Staph	$2.10X10^{2}$
	S. aureus	API Staph	$2.29X10^{3}$
	Bacillus sp.	16S	$6.00X10^{1}$
	Corynebacterium accolens	16S	$1.70X10^{2}$
	Gemella haemolysans	16S	$2.00X10^{1}$
260	*S. epidermidis*	16S	$1.39X10^{3}$
	Micrococcus sp.	API Staph	$4.00X10^{1}$
	S. xylosus	API Staph	$1.22X10^{3}$
	Corynebacterium smegmentosum	16S	$1.39X10^{3}$
	Enterobacter aerogenes	16S	$1.20X10^{2}$
282	*S. epidermidis*	API Staph	$1.10X10^{4}$
	Micrococcus luteus	API Staph	$4.00X10^{1}$
	S. xylosus	API Staph	$8.00X10^{1}$
	Streptococcus sp.	API Staph	$6.60X10^{4}$
	Raoultella sp.	16S	$4.00X10^{1}$
251	*S. epidermidis*	API Staph	$2.55X10^{4}$
	S. capitis	API Staph	$4.00X10^{1}$

	S. xylosus	API Staph	$4.00X10^{1}$
	Bacillus sp.	16S	$4.00X10^{1}$
261	*S. hominis*	API Staph	$4.41X10^{4}$
	Micrococcus luteus	16S	$2.00X10^{1}$
290	*Micrococcus luteus*	16S	$1.60X10^{4}$
	S. capitis	16S	$9.10X10^{4}$
252	*S. hominis*	API Staph	$4.60X10^{2}$
	S. capitis	API Staph	$2.00X10^{1}$
	Corynebacterum accolens	16S	$2.23X10^{3}$
	E. coli	16S	$2.00X10^{1}$
277	*Micrococcus* sp.	API Staph	$1.00X10^{3}$
	S. aureus	API Staph	$4.50X10^{3}$
	Corynebacterium sp.	16S	$1.31X10^{5}$
	Streptococcus pyogenes	16S	$1.37X10^{5}$
279	*S. capitis*	API Staph	$2.00X10^{1}$
	S. aureus	API Staph	$9.40X10^{3}$
	Streptococcus pyogenes	16S	$1.09X10^{4}$
271	*S. hominis*	API Staph	$4.00X10^{2}$
	S. aureus	API Staph	$4.80X10^{3}$
	S. xylosus	API Staph	$5.30X10^{2}$
	Streptococcus sp.	16S	$4.90X10^{2}$
330	*S. hominis*	API Staph	$1.12X10^{4}$
	S. aureus	API Staph	$1.03X10^{4}$
	S. pasteuri	16S	$2.00X10^{2}$

Table 8.1 Microbial composition of each community. The exact composition of each community with the method by which each taxonomically distinct unit was identified, either by API Staph (BioMérieux, Marcy-l'Etoile, France) or 16S rDNA sequencing (method 2.5). Species level identification was assigned where possible. The density represents the mean number of colonies sampled from an individual nose.

Species	Genus	Family	Order	Class	Phylum
C. accolans	*Corynebacterium*	Corynebacteriaceae	Actinomycetales	Actinobacteria	Actinobacteria
C. coyleae	*Corynebacterium*	Corynebacteriaceae	Actinomycetales	Actinobacteria	Actinobacteria
C. fastidium	*Corynebacterium*	Corynebacteriaceae	Actinomycetales	Actinobacteria	Actinobacteria
C. macginleyi	*Corynebacterium*	Corynebacteriaceae	Actinomycetales	Actinobacteria	Actinobacteria
C. propinquum	*Corynebacterium*	Corynebacteriaceae	Actinomycetales	Actinobacteria	Actinobacteria
C. pseudodipthericum	*Corynebacterium*	Corynebacteriaceae	Actinomycetales	Actinobacteria	Actinobacteria
C. segmentosum	*Corynebacterium*	Corynebacteriaceae	Actinomycetales	Actinobacteria	Actinobacteria
K. kristinae	*Kocuria*	Micrococcaceae	Actinomycetales	Actinobacteria	Actinobacteria
Unidentified	*Micrococcus*	Micrococcaceae	Actinomycetales	Actinobacteria	Actinobacteria
M. luteus	*Micrococcus*	Micrococcaceae	Actinomycetales	Actinobacteria	Actinobacteria
Unidentified	*Bacillus*	Bacillaceae	Bacillales	Bacilli	Firmicutes
B. simplex	*Bacillus*	Bacillaceae	Bacillales	Bacilli	Firmicutes
G. haemolysans	*Gemella*	Staphylococcaceae	Bacillales	Bacilli	Firmicutes
S. aureus	*Staphylococcus*	Staphylococcaceae	Bacillales	Bacilli	Firmicutes
S. capitis	*Staphylococcus*	Staphylococcaceae	Bacillales	Bacilli	Firmicutes
S. equorum	*Staphylococcus*	Staphylococcaceae	Bacillales	Bacilli	Firmicutes
S. haemolyticus	*Staphylococcus*	Staphylococcaceae	Bacillales	Bacilli	Firmicutes
S. lentus	*Staphylococcus*	Staphylococcaceae	Bacillales	Bacilli	Firmicutes
S. lugdunensis	*Staphylococcus*	Staphylococcaceae	Bacillales	Bacilli	Firmicutes
S. pasteuri	*Staphylococcus*	Staphylococcaceae	Bacillales	Bacilli	Firmicutes
S. saprophiticus	*Staphylococcus*	Staphylococcaceae	Bacillales	Bacilli	Firmicutes
S. schleiferi	*Staphylococcus*	Staphylococcaceae	Bacillales	Bacilli	Firmicutes
S. sciuri	*Staphylococcus*	Staphylococcaceae	Bacillales	Bacilli	Firmicutes
S. simulans	*Staphylococcus*	Staphylococcaceae	Bacillales	Bacilli	Firmicutes
S. warneri	*Staphylococcus*	Staphylococcaceae	Bacillales	Bacilli	Firmicutes
S. xylosus	*Staphylococcus*	Staphylococcaceae	Bacillales	Bacilli	Firmicutes
S.epidermidis	*Staphylococcus*	Staphylococcaceae	Bacillales	Bacilli	Firmicutes
S.hominis	*Staphylococcus*	Staphylococcaceae	Bacillales	Bacilli	Firmicutes
E. faecalis	*Enterococcus*	Enterococcaceae	Lactobacillales	Bacilli	Firmicutes
Unidentified	*Streptococcus*	Streptococcaceae	Lactobacillales	Bacilli	Firmicutes
S. pyogenes	*Streptococcus*	Streptococcaceae	Lactobacillales	Bacilli	Firmicutes
E. aerogenes	*Enterobacter*	Enterbacteriaceae	Enterobacteriales	Gamma Proteobacteria	Proteobacteria
E. coli	*Escherichia*	Enterbacteriaceae	Enterobacteriales	Gamma Proteobacteria	Proteobacteria
Unidentified	*Raoultella*	Enterbacteriaceae	Enterobacteriales	Gamma Proteobacteria	Proteobacteria
C. freundii	*Citrobacter*	Enterobacteriaceae	Enterobacteriales	Gamma Proteobacteria	Proteobacteria
C. koseri	*Citrobacter*	Enterobacteriaceae	Enterobacteriales	Gamma Proteobacteria	Proteobacteria
M. nonliquiformans	*Morexella*	Morexellaceae	Pseudomonadales	Gamma Proteobacteria	Proteobacteria

Table 8.2. Species classes isolated from this study

Presumptive ID	BEN ID	Participant ID	Colony Morphology
S. hominis	BEN002	SWB281	3mm yellow matt
S. epidermidis	BEN004	SWB281	0.5mm white
Micrococcus sp	BEN007	SWB250	2mm brown pale
S. capitis	BEN010	SWB250	5mm cream pale
S. epidermidis	BEN011	SWB250	0.7mm opaque
Coryne	BEN012	SWB250	0.5mm grey
Micrococcus	BEN013	SWB291	0.3mm white
S. aureus	BEN014	SWB291	2mm cream yellow
S.epidermidis	BEN015	SWB291	5mm white
S. warneri	BEN016	SWB291	5mm light yellow
Coryne	BEN019	SWB265	0.3mm white
S. warneri	BEN020	SWB265	4mm white
S. epidermidis	BEN021	SWB265	4mm white
S. capitis	BEN022	SWB265	1mm bright white
S. epidermidis	BEN025	SWB293	2mm white; entire; glossy
S. hominis	BEN026	SWB293	3mm yellow; entire; glossy
S. aureus	BEN028	SWB284	
S. epidermidis	BEN030	SWB284	
Corynebacterium coyleae	BEN031	SWB284	
S. aureus	BEN032	SWB259	7mm pale yellow centre
S. epidermidis	BEN033	SWB259	5mm white
S. aureus	BEN034	SWB270	2mm yellow middle
S. epidermidis	BEN035	SWB270	3mm white; glossy; entire
S. aureus	BEN039	SWB258	0.3mm cream/grey
S. epidermidis	BEN040	SWB258	2mm cream/grey
S. capitis	BEN041	SWB258	1.5mm bright white
S. epidermidis	BEN043	SWB255	4mm right white
S. aureus	BEN044	SWB255	4mm bright yellow
M. Luteus	BEN046	SWB255	1mm pale yellow
S. warnerei	BEN047	SWB255	3mm pale yellow
S. epidermidis	BEN048	SWB272	2mm cream white
Citrobacter koseri	BEN049	SWB272	5mm pale yellow/orange glossy entire
uncultivable	BEN050	SWB272	pinpoint colonies on MSA
S. aureus	BEN051	SWB299	
Citrobacter freundii	BEN052	SWB299	5mm raised yellow un-entire colonies (on blood)
S. hominis	BEN053	SWB299	2mm bright yellow; entire; glossy
S. epidermidis	BEN056	SWB299	4mm white; entire; glossy
S. epidermidis	BEN057	SWB296	0.3mm white
Micrococcus	BEN058	SWB296	2mm light yellow; glossy
S. aureus	BEN059	SWB296	4mm deep yellow; entire; glossy
Bacillus	BEN061	SWB296	2mm cream; entire; matt
Coryneform	BEN062	SWB277	1mm white

Micrococcus	BEN063	SWB277	1mm; light brown; glossy; enthire
Kocuria sp	BEN064	SWB277	1mm; light yellow; entire; glossy
S. aureus	BEN065	SWB277	5mm cream; entire; glossy
Streptococcus pyogenes	BEN066	SWB277	0.5mm; clear; entire
S. saprophyticus	BEN067	SWB286	
S. aureus	BEN068	SWB286	2mm; grey centre; glossy (on blood)
Kocuria kristinae	BEN069	SWB286	2mm; light yellow; glossy; entire
uncultured	BEN070	SWB286	opaque pinpoint
S. aureus	BEN071	SWB286	3mm large yellow centre; grey outside
Coryne	BEN072	SWB289	0.8mm white
Streptococcus pyogenes	BEN073	SWB289	2mm grey; entire; glossy (horse blood)
S. epidermidis	BEN074	SWB289	2mm white/cream; entire (horse blood)
Streptococcus pyogenes	BEN075	SWB279	1mm cream; glossy; entire
S. aureus	BEN076	SWB279	3mm; white; glossy; entire
S. capitis	BEN077	SWB279	0.7mm bright yellow
S. epidermidis	BEN078	SWB274	5mm white; entire; glossy
S. aureus	BEN079	SWB274	2mm; white; entire; glossy
S. pasteuri	BEN080	SWB274	3mm yellow; entire; glossy
S. epidermidis	BEN082	SWB263	2mm; white; entire; glossy
S. epidermidis	BEN084	SWB297	5mm white; gloss; entire
S. pasteuri	BEN085	SWB297	5mm grey/yellow; glossy; entire
Moraxella nonliquefacians	BEN088	SWB297	1mm opaque
Coryne	BEN090	SWB267	1mm grey/white; entire; glossy
S. epidermidis	BEN092	SWB267	2mm bright white; glossy; entire
S. sciuri	BEN094	SWB267	5mm large matt; off cream (BHI)
S. epidermidis	BEN095	SWB269	4mm white; glossy; entire
Coryneform	BEN096	SWB269	2mm off cream; glossy; entire
S. capitis	BEN098	SWB269	Bright white; entire; glossy (BHI)
S. Pasteuri	BEN099	SWB269	off yellow; glossy; entire (blood)
S. epidermidis	BEN101	SWB275	2m off white/grey yellow centre
S. warneri	BEN102	SWB275	2mm yellow entire gloss
coryne	BEN103	SWB275	Pinpoint
S. schleiferi	BEN104	SWB275	2m bright white
S. epidermidis	BEN105	SWB288	2mm white; gloss; entire
S. aureus	BEN106	SWB288	2mm pale yellow; gloss; entire
Actinobacterium	BEN107	SWB288	<0.5mm pale yellow
M. Luteus	BEN108	SWB288	1mm bright yellow
Bacillus simplex	BEN109	SWB288	5mm rough brown un-entire convex (blood)
S. xylosus	BEN110	SWB282	2mm white; glossy; entire
Raoultella sp	BEN111	SWB282	4mm cream mucoid
M.luteus	BEN112	SWB282	Pale yellow; glossy; entire
Streptococcus	BEN113	SWB282	<0.5mm

S. epidermidis	BEN114	SWB282	Pale brown; glossy entire (blood)
S. epidermidis	BEN115	SWB294	3mm white entire glossy
S. aureus	BEN116	SWB294	4mm off yellow centre, glossy; entire
S. equorum	BEN117	SWB294	2mm bright yellow, glossy; entire
S. hominis	BEN119	SWB252	3mm cream white; glossy; entire
Coryne	BEN120	SWB252	Pinpoint
E. Coli	BEN121	SWB252	5mm rough mucoid brown
S. capitis	BEN122	SWB252	White with slight (barely noticeable) yellowing round the edges
S. aureus	BEN123	SWB298	4mm gold coin; smooth; glossy; entire
S. epidermidis	BEN124	SWB298	3mm cream; glossy; entire
Strep	BEN125	SWB298	<1mm opaque (on blood agar)
S. hominis	BEN126	SWB290	3mm white, glossy; entire
S. capitis	BEN127	SWB290	1mm cream; glossy entire
Micrococcus luteus	BEN128	SWB290	1mm yellow; matt; entire
S. epidermidis	BEN129	SWB285	3mm cream; glossy; entire
S. aureus	BEN130	SWB285	3mm dark yellow; glossy; entire
S. epidermidis	BEN131	SWB285	1mm white; glossy; entire
Actinobacterium	BEN133	SWB285	<1mm
M. Luteus	BEN134	SWB285	
S. epidermidis	BEN136	SWB276	<1mm
S. aureus	BEN137	SWB253	2mm yellow; glossy; entire
S. epidermidis	BEN138	SWB253	1mm white
Bacillus	BEN139	SWB253	3mm brown, rough edge; matt
Coryne	BEN140	SWB253	<1mm
Gemella haemolysans	BEN141	SWB253	1mm grey (big a - haem)
S. epidermidis	BEN142	SWB273	3mm bright white; glossy; entire
Coryne	BEN143	SWB273	1mm cream, glossy; entire
Streptococcus/Streptobacillus	BEN144	SWB273	<1mm opaque
S. haemolyticus	BEN145	SWB273	4mm cream; glossy; entire
S. lentus	BEN146	SWB273	3mm gloopy blob on top of confluent growth
S. hominis	BEN150	SWB261	3mm cream white
M. Luteus	BEN151	SWB261	1mm bright yellow
S. xylosus	BEN153	SWB260	3mm white; glossy; entire
Micrococcus	BEN154	SWB260	1mm yellow; glossy; entire
S. epidermidis	BEN155	SWB260	2mm white; v glossy; entire
Actinobacterium	BEN156	SWB260	<1mm opaque
Enterobacter aerogenes	BEN157	SWB260	3mm cream; large haem on SB
S. epidermidis	BEN158	SWB251	3mm cream white; glossy; entire
bacillus	BEN159	SWB251	10mm cream; matt; un-entire
S. capitis	BEN160	SWB251	3mm pale yellow
S. xylosus	BEN161	SWB251	2mm light brown colonies (on blood)

S. epidermidis	BEN164	SWB268	4mm grey; glossy; entire; haem (choc agar)
S. lugdunesis	BEN166	SWB268	<1mm
Micrococcus	BEN168	SWB268	2mm bright yellow
S. aureus	BEN169	SWB264	3mm yellow gold coin
S. epidermidis	BEN170	SWB264	2mm pale grey
S. aureus	BEN171	SWB271	4mm gold coin
S. hominis	BEN172	SWB271	3mm white; glossy; entire
S. xylosus	BEN173	SWB271	2mm cream grey; glossy; entire
Streptococcus	BEN174	SWB271	<1mm (on sheep blood)
S. hominis	BEN175	SWB287	<1mm
S. epidermidis	BEN176	SWB287	3mm grey/cream; glossy; entire
S. aureus	BEN177	SWB287	1mm grey outside/cream centre; glossy; entire
ascomycete	BEN178	SWB256	<1mm on sheep blood
S. epidermidis	BEN180	SWB256	3mm grey
S. aureus	BEN184	SWB278	4mm grey/light yellow; glossy; entire
S. epidermidis	BEN185	SWB278	4mm grey; glossy; entire
***Corynebacterium* sp**	BEN186	SWB278	<1mm
S. warneri	BEN187	SWB278	4mm light yellow; glossy; entire
S. epidermidis	BEN188	SWB262	3mm grey; glossy; entire
S. lugdunensis	BEN189	SWB262	2.5mm white; glossy; entire
M. Luteus	BEN191	SWB262	2mm yellow
Coryne	BEN192	SWB262	<1mm blood plates
S. epidermidis	BEN193	SWB295	3mm grey; glossy; entire
Corynebacterium accolens	BEN196	SWB295	<1mm
S. epidermidis	BEN197	SWB254	3mm grey; glossy; entire
***bacillus* sp**	BEN198	SWB254	5mm gloopy; grey; un-entire
Corynebacterium macginleyi	BEN201	SWB254	<1mm
M.luteus	BEN202	SWB254	1mm bright yellow; glossy; entire
S. epidermidis	BEN203	SWB280	3mm; cream grey, glossy; entire
Coryne	BEN204	SWB280	1mm pale pink/cream; glossy; entire
S. aureus	BEN207	SWB266	4mm; gold coin; glossy; entire
S. epidermidis	BEN208	SWB266	3mm off white; glossy; entire
Micrococcus	BEN212	SWB292	2mm bright yellow; glossy; entire
S. epidermidis	BEN213	SWB292	2mm white; glossy; entire
M. Luteus	BEN214	SWB292	1mm Light yellow; glossy; entire
S. aureus	BEN215	SWB292	<1mm dark yellow
S. aureus	BEN216	SWB330	3mm white; glossy; entire
S. hominis	BEN217	SWB330	<1>0.5 opaque
S. pasteuri	BEN219	SWB330	2mm yellow; glossy; entire
S. aureus	BEN220	SWB327	3mm gold coin; glossy; entire
uncultivable	BEN222	SWB327	<1mm
S. epidermidis	BEN224	SWB340	3mm white (haem on blood)
Enterococcus feacalis	BEN226	SWB340	1mm grey (black haem)

S.hominis	BEN227	SWB340	2mm yellow; glossy; entire
S. aureus	BEN228	SWB334	3mm Gold coin; glossy; entire
S. lugdunensis	BEN229	SWB334	2mm Bright white; glossy; entire
S. epidermidis	BEN230	SWB334	2mm Grey; glossy; entire
S. epidermidis	BEN232	SWB315	1mm cream grey; glossy; entire
S. aureus	BEN234	SWB315	1mm dark yellow
S. warneri	BEN235	SWB315	2mm light yellow; glossy; entire
uncultured	BEN236	SWB315	<1mm
S. capitis	BEN237	SWB313	2mm Cream; Glossy; entire
Streptococcus mitis	BEN239	SWB313	1mm opaque (black haem sheep)
S. epiderimidis	BEN240	SWB313	<1mm
Brevidobacterium	BEN241	SWB313	
S. epidermidis	BEN243	SWB337	3mm white; glossy; entire
S. epidermidis	BEN244	SWB337	3mm white + haem
S. capitis	BEN245	SWB337	2mm bright white
S. epidermidis	BEN246	SWB337	2mm yellow; matt; entire
S. aureus	BEN247	SWB324	3mm Gold coin; glossy; entire
S. epidermidis	BEN248	SWB324	2mm grey; glossy; entire
S. lugdunensis	BEN250	SWB324	bright white
Streptococcus sp	BEN251	SWB324	<1mm
S. epidermidis	BEN252	SWB319	2mm grey (on sheep)
Enterobacter aerogenes	BEN253	SWB319	2mm grey; glossy; entire
Streptococcus sp	BEN254	SWB319	<1mm
S. hominis	BEN255	SWB301	1.5mm light yellow;
S. simulans	BEN256	SWB301	4mm cream; glossy; entire
S. epidermidis	BEN257	SWB301	2mm grey, glossy; entire
Uncultured	BEN259	SWB301	
S. epidermidis	BEN263	SWB257	4mm bright white; glossy; entire
Coryne	BEN264	SWB257	1mm cream; glossy; entire
Streptococcus sp	BEN265	SWB257	<1mm
Bacillus	BEN266	SWB257	Brown gloopy clumps 4mm (appearance on blood)
S. epidermidis	BEN271	SWB283	2mm cream; glossy; entire
Micrococcus	BEN272	SWB283	1mm grey; glossy; entire
Coryne	BEN273	SWB283	<1mm
S.aureus	BEN275	SWB283	1.5mm light orange/ cream; glossy; entire
M. Luteus	BEN276	SWB283	1mm bright yellow; glossy; entire

Table 8.3. Identification and colony morphology of strains from this study.

Chapter 9: References

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